LANE MEDICAL LIBRARY STANFORD
F601 .W16w 1929 OLD
Enzyme actions and properties / by Ernst
24503110955

LANE



MEDICAL LIBRARY

LEVI COOPER LANE FUND

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305


Digitized by the Internet Archive
in 2025

https://archive.org/details/enzymeactionspro0000erns

ENZYME ACTIONS AND PROPERTIES

ENZYME ACTIONS AND PROPERTIES

BY

ERNST WALDSCHMIDT-LEITZ

Institut für Biochemie, Deutsche Technische Hochschule, Prag.
Formerly at the University of Munich

TRANSLATED AND EXTENDED

BY

ROBERT P. WALTON

Department of Organic Chemistry, Columbia University

NEW YORK

JOHN WILEY & SONS, INC.

LONDON: CHAPMAN & HALL, LIMITED

1929

Technical Book Company
525
Market St.
GArfield 2617

COPYRIGHT, 1929,
BY ROBERT P. WALTON

Printed in U. S. A.

PRESS OF
BRAUNWORTH & CO., INC.
BOOK MANUFACTURERS
BROOKLYN, NEW YORK

F601
W16W
1929

DEDICATED TO

GEHEIMRAT

PROF. DR. R. WILLSTÄTTER

IN HONOR AND APPRECIATION

71149

MOTTO

The study of enzymic processes appears to me to be destined to enlarge also our views with respect to the molecular structure of complicated organic compounds.—*Zeit. für physiologische Chemie* 26, p. 68 (1898).

EMIL FISCHER.

PREFACE

The phenomena of enzyme actions represent a signally prominent aspect of organic chemistry. The mechanisms of those chemical transformations taking place in the life processes of plants and animals are effected principally by the action of enzymes. In fact, it has been whimsically said that life is just one enzyme reaction after another. Certain it is that the significance of enzyme transformations in biological processes is to be regarded as of the first magnitude. Beyond the purely biochemical aspects, enzyme reagents have been used to advantage in elucidating the constitution of high molecular organic compounds and also in the preparation of easily alterable, organic substances. Among the purely utilitarian phases of enzyme study may be mentioned the application of enzymes to medical diagnosis, to the control of diseases and to the performance of numerous industrial operations. These latter phases offer a particularly attractive incentive to the further application of enzymic principles.

Since the first clearly characterized description of enzyme activity by Kirchhoff in 1814, an enormous amount of work has been directed toward the study of this field. Environmental conditions of action, preservation and destruction have been determined for a large number of enzymic agents, methods of purification have been developed, procedures of measurement have been evolved and in some cases even experimental syntheses have been attempted. The modern worker in the field of enzymes inherits a voluminous accumulation of previous experiences. The value of such a heritage, however, is to be accepted with reservations. The extensive distribution of enzymes and the diversity of objectives motivating their study have injected into the literature many results which are incapable of correlation.

The controversial aspect of the field was particularly marked some ten years past. Accordingly, at this period it was an event of singular interest when enzyme studies on an extensive scale

were initiated by the Willstätter school. The previous success of R. Willstätter and associates in determining the constitution of chlorophyll and other highly complex, natural, organic materials had gained wide recognition. The Nobel Prize in chemistry, among other awards, attested to the value and significance of such achievements. An incidental feature of unique interest was the fact that this research campaign was being conducted in Munich, where E. Buchner first accomplished the classical separation of the zymase complex from living yeast cells. This battalion of student researchers, directed by that master of organic chemistry, R. Willstätter, was working on the same site at which one of the most significant events of enzyme chemistry had been enacted.

It was generally hoped that the chemical constitution of some typical enzymes would be determined in the course of these researches. More than a decade has now passed since the inception of this work and the constitution of enzymes is as yet a matter of general speculation. Nevertheless, the contributions from the Munich laboratories have been of profoundest significance to the progress of enzyme studies. A fundamental advance in preparative technique has been introduced by the application of strictly quantitative controls. That is, the total enzyme yield (relative activity *times* quantity of preparate) has been considered as a criterion of preparative success in contrast to the older practice of considering merely the activity degree of a given preparate. In substance, the consideration of " total yields " has been instituted in enzyme chemistry; such a consideration has long been conventional in preparative organic chemistry but had hitherto not been consistently applied to preparative enzyme studies. The use of this guiding principle as a criterion for the success of each separate purification stage has permitted an unprecedented degree of success in these preparative experiments; in some cases, as by adsorption, the ultimate limits of these methods have probably been attained. Further, the preparation of integrally pure enzymes by selective methods of separation has permitted the establishment of sharply marked specificity boundaries, which had previously been indefinite or else entirely incorrect. Also, by working with a large number of typical enzymes under the uniform conditions existent in one laboratory, many points of correlation have been made possible and many inaccurate reports

of the previous literature have been decisively disproven. Without question these researches have developed a more unified and advanced conception of enzyme nature, a conception, further, which has been derived upon a strictly experimental basis.

The material within this volume constitutes a general treatment of enzyme action and properties as interpreted principally from the viewpoint of the Willstätter school. Prof. E. Waldschmidt-Leitz, the active associate of R. Willstätter, has given a condensed description of the most important principles of enzyme chemistry and of the special characteristics of the most prominent enzymes. The work has not been attempted as an exhaustive compendium of enzyme literature but rather as a concise presentation of the most fundamental principles and best accepted characteristics of enzyme action.

The translation of this volume has been prompted by the obvious value of the work and by the eminent need for a modern, authoritative treatment of the subject in the English language. It is considered that the volume constitutes a general treatment which will be of value not only to researchers who are concerned directly with the study of the field but also to those who have only a conjunctive interest in the subject. Students of medicine, biochemistry, physiology, botany and related fields should find this volume of service in acquiring an orientated conception of the modern status of enzyme studies. Although many controversial aspects of the subject are presented, the highly authoritative viewpoint from which the volume has been developed should commend it to the general reader.

The original German text has been considerably extended, although the additions have been principally from the viewpoint of the Willstätter school. The purpose of the modifications has been primarily to bring the context of the volume up to the present date rather than to impose any particular viewpoint of the translator. This revision and extension has been carried out with the cooperation of the author and warmest thanks are due him for his careful consideration in this work.

The translator, further, wishes to acknowledge the assistance of several of his friends and associates. Dr. Maxwell P. Schubert, Dr. Harry Sobotka and Dr. Ph. Papadakis have read portions of the manuscript and made numerous suggestions. Miss May Tevis has been employed to assist in the translation, to read proof

and to prepare the author index. In particular the translator is grateful for the valuable counsel and advice given by Prof. John M. Nelson of Columbia University, who suggested that this work be undertaken.

R. P. W.

New York City,
August, 1928.

CONTENTS

GENERAL SECTION

PAGES

PREFACE vii

ABBREVIATIONS xvii

I. DEVELOPMENT OF THE FERMENT CONCEPT 1– 6

"Fermentatio," 1. Era of Liebig's Researches, 1. Vitalistic Assumption of Pasteur, 2. Definition of Enzymes, 3. Material Nature of Enzymes, 3.

II. ENZYMES AS COLLOIDS 7– 9

Colloidal Characteristics, 7. Particle Size, 7. Diffusion Velocities and Molecular Weights, 8.

III. ENZYMES AS ELECTROLYTES 10– 23

Significance of Hydrogen-Ion Concentration, 10. Buffers, 10. Activity-*p*H Curves, 11. Disturbance of *p*H Dependence, 12. Enzymes as Amphoteric Electrolytes, 14. Ionization of the Substrate, 16. Migration in Electrical Fields, 17. Influence of Associated Materials, 17. Electro-chemical Adsorption, 18. Adsorption Behavior of Enzymes, 19. Preparative Adsorption Methods, 20. Adsorption Curves, 21. Elution, 23.

IV. ENZYMIC KINETICS 24– 59

Mass Action Law, 24. Mono-molecular Reactions, 24. Varying Kinetics of Saccharase Action, 26. Enzyme Quantity and Reaction Velocity, 27. The Schütz Rule, 28. Enzyme-Substrate Compounds, 29. Substrate Concentration and Reaction Velocity, 30. Activity-*p*S Curves, 32. Dissociation Constants, 33. Affinity Variations, 34. Formation and Decomposition of the Enzyme-Substrate Compound, 35. Influence of Reaction Products, 36. Differing Types of Inhibition, 39. Reaction Equation of Saccharase Action, 40. Action of Hydrogen-Ions, 42. Temperature Relations, 43. Temperature Inactivation, 43. Enzyme and H-Ion Catalyses, 45. Mechanism of Enzyme Actions, 47. Application of the Mass Action Law, 48. The Specific Reactive Group, 50. Euler's Conception, 51. Enzymic Equilibria and Syntheses, 52. Equilibrium Point of Enzyme Reactions, 57.

PAGES

V. Enzymic Reaction Systems (Activation and Inhibition)..... 60– 69

Zymogens, 60. Activation by Inorganic Ions, 60. Specific Activation, 62. Non-Specific Activations and Inhibitions, 64. Toxic Influence of Heavy Metal Salts, 67. Equivalent Weights of Enzymes, 67. Inactivation by Amines, 68. Other Toxic Agents, 68. Anti-Enzymes, 69.

VI. Enzymic Specificity..... 70– 73

Specificity of Reaction Paths, 70. Absolute and Relative Specificities, 71. Stereochemical Specificity, 73.

VII. Detailed Procedures in Preparative Enzyme Chemistry 74–100

1. Quantitative Determination..... 74– 79

Activity, the Criterion of Concentration, 74. Measurement Scales, 75. Characteristic Examples, 78.

2. Occurrence and Formation of Enzymes..... 79–89

Distribution and Occurrence, 79. Exo- and Endo-Enzymes, 80. Liberation of the Enzymes, 80. Solubility Gradations, 82. Enzyme Formation and Secretions in Animal Organisms, 84. Enzyme Formation in One-celled Organisms, 85. Further Enrichment of Invertase in Yeasts, 87. Adaptation of Yeast to Various Substrates, 88.

3. Technique of Preparation and Purification..... 89–100

Solution after Destruction of Cell-structure, 89. Autolysis, 89. Drying and Subsequent Extraction, 90. Dialysis, 91. Precipitation, 91. Adsorption of Enzymes, 92. Early Attempts with Adsorption Methods, 94. Willstätter's Separation of Pancreatic Enzymes, 94. Recognition and Differentiation of Adsorption Agents, 96. Alternate Use of Various Adsorption Agents, 98. Limitations of the Adsorption Procedures, 99. Purification of Insoluble Enzymes, 99.

SPECIAL SECTION

I. Esterases..... 101–124

1. Animal Esterases..... 101–116

(*a*) Pancreas Lipase..... 101–108

Methods of Determination, 102. Preparative Purification, 105. Adsorption Behavior, 106.

(*b*) Stomach Lipase..... 108–110

Hydrogen-Ion Dependence, 108. Measurement, 109. Increase in Degree of Purity, 109

(*c*) Liver Esterase..... 110–114

Kinetics, 110. Comparison with Pancreas Lipase, 111. Purification, 111. Induction Period, 112. Affinity to Keto-Carboxylic Acid Esters, 113.

PAGES

THE SPECIFICITY OF ANIMAL ESTERASES 114–116
Relative Specificity, 114. Stereochemical Specificity, 114. Toxic Susceptibility, 116.

2. PLANT ESTERASES 116–124
(*a*) Ricinus Lipase 116–120
Working Conditions, 117. Procedure for Purifications, 118. Alteration During Germination, 120.
(*b*) Chlorophyllase 121–122
Action, 121. Analytical Procedure, 121.
(*c*) Tannase 122–123
Occurrence and Action, 122. Kinetics, 122.
(*d*) Phosphatase, Sulfatase 123–124

II. PROTEASES AND PEPTIDASES 125–167

DETERMINATION OF PROTEOLYTIC DEGRADATION 125–128
Methods Based on Determination of Unaltered Substrate, 125. Physical Methods Based on Determination of Cleavage Products, 126. Chemical Methods Based on Determination of Cleavage Products, 127.

1. TRUE PROTEASES 129–155
(*a*) Pepsin, Lab 129–139
Formation, 129. Supposed Inactive Pre-Stage, 129. Hydrogen-Ion Dependence, 129. Kinetics, 130. Specificity and Mode of Action, 131. Preparative Purification, 135. Plastein Formation, 136. Milk Coagulative Action, 137. Proteases in the Spleen, 138.
(*b*) Trypsin 139–148
Occurrence, 139. Hydrogen-Ion Dependence, 139. Kinetics, 140. Activation of Enterokinase, 140. Spontaneous Formation of Enterokinase, 141. Preparation and Properties of Enterokinase, 142. Measurement of Trypsin Action, 143. Preparation of Trypsin, 144. Specificity of Trypsin, 146.
(*c*) Thrombin 148–149
(*d*) Proteases of Higher Plants 149–153
Papain, 149. Bromelin, 151. Protease of Pumpkin, 152. Hydrogen-Ion Dependence, 152. Associated Ereptase, 153. Malt Proteases, 153.
(*e*) Yeast Proteases 153–155

2. PEPTIDASES 155–161
Intestinal Erepsin, 155. Identity of Pancreas and Intestinal Erepsin, 156. Hydrogen-Ion Dependence of Erepsin, 157. Kinetics, 157. Preparative Purification, 158. Specificity of Peptidases, 159.

3. MECHANISM OF PROTEIN AND PEPTIDE DEGRADATIONS 161–165
Peptidase Action, 161. Protase Action, 164.

CLASSIFICATION OF THE PROTEASES 166–167

PAGES

III. AMINOACYLASES 168–177

1. HISTOZYME 168–169

Occurrence and Actions, 168. Solubility, 169.

2. ARGINASE 169–172

Action, 169. Occurrence and Relation to Sexual Activity, 170. Determination, 170.

3. UREASE 172–177

Occurrence and Importance, 172. Hydrogen-Ion Dependence, 193. Supposed Activations, 173. Kinetics, 174. Mechanism of Urease Action, 176. Preparative Purification, 177.

IV. CARBOHYDRASES 178–209

1. HEXOSIDASES 178–198

(*a*) Fructosidases, Saccharase 178–188

Special Characteristics, 178. Measurement, 178. Occurrence, 180. Specificity, 180. Liberation of Yeast Saccharase, 181. Autolysis Procedures, 182. Preparative Purifications, 183. Composition of Saccharase Preparates, 187.

(*b*) α-Glucosidases: Maltase 188–193

Occurrence, 188. Hydrogen-Ion Dependence, 189. Kinetics, 189. Scale for Measurement, 190. Specificity, 190. Maltase Solutions from Yeast, 190. Separation from Saccharase, 192.

(*c*) β-Glucosidases: Emulsin 193–196

Reaction Course of Amygdalin Cleavage, 193. Kinetics, 195. Measurement, 195. Preparative Separation, 196.

(*d*) Galactosidases: Lactase, Melibiase 196–198

2. POLYASES 198–209

(*a*) Amylases 198–208

Occurrence, 198. Measurement, 198. Salt Activation and *p*H Dependence, 199. Kinetics, 201. Activity Scales, 202. Liquefying and Saccharifying Actions of Amylases, 203. Alpha and Beta Amylases, 205. Purification, 205. Purification of Malt Amylase, 207.

(*b*) Inulase, Lichenase, Cellulase 208–209

V. CATALASES 210–215

Characteristics and Significance in Respiration, 210. Hydrogen-Ion Dependence, 211. Kinetics, 211. Catalytic Nature and Units of Measurement, 212. Preparation, 213.

VI. PEROXYDASES 216–225

Distribution and Function, 216. Procedures and Scales for Measurement, 216. Reliability of the Purpurogallin Method, 218. Preparative Procedures, 219. Dialysis of the Plant Material, 219. Precipitation with Oxalic Acid, 220. Fractional Extraction, 220. Precipitation with Mercury Salts and Tannic

PAGES

Acid, 220. Adsorption by Alumina and Kaolin, 221. Spontaneous Increase of Peroxidase Activity, 222. Increase in Purity-Degree, 223. Relation of Color and Activity, 223. Iron Content, 224. Peroxidasic Action of Oxyhaemoglobin, 224.

VII. Oxydases 226–232

Mechanism of Biological Oxidations According to Bach, 226. Hydrogen Activation According to Wieland, 227. The Iron Cycle According to Warburg, 229. Action of Glutathione, 230. Purine Oxydases, 231. Tyrosinase, 232.

VIII. Fermentation Enzymes 233–241

The Enzyme Complex "Zymase," 233. The Heat Resistant, "Co-Zymase," 233. Kinetics of Alcoholic Fermentation, 234. Adaptation of Zymase to Various Substrates, 236. Direct Fermentation of Disaccharides, 237. Mechanism of Alcoholic Fermentation and Significance of Phosphoric Acid, 237. Formation of Lactic Acid, 238. Formation of Pyroracemic Acid and Acetaldehyde, 239. Acetic Acid Fermentation, 240. Synthetic Fermentations, 241.

Author Index 243

Subject Index 249

ABBREVIATIONS

A	Liebigs Annalen der Chemie
A. Ch.	Annales de chimie et de physique
Am.	American Chemical Journal
Am. Soc.	Journal of the American Chemical Society
Ann. d. Physik	Annalen der Physik
Ann. Inst. Pasteur	Annales de l'Institut Pasteur
Arch. de Physiol.	Archive de Physiologie
Arch. exp. Pathol. u. Pharmakol.	Archiv. für experimentelle Pathologie und Pharmakologie
Arch. int. de Physiol.	Archives internationales de Physiologie
B.	Berichte der Deutschen Chemischen Gesellschaft
Ber. d. Deutsch. Pharm. Ges.	Berichte der Deutschen Pharmazeutischen Gesellschaft
Biochem. Jl.	Biochemical Journal
Bio. Z.	Biochemische Zeitschrift
Bl.	Bulletin de la Société Chimique de France
C.	Chemisches Zentralblatt
Chem. Weekbl.	Chemisch Weekblad
C. r.	Comptes rendus de l'Académie des Sciences
D. Arch. klin. Med.	Deutsches Archiv für klinische Medizin
Ergebn. d. Physiol.	Ergebnisse der Physiologie
Fermentf.	Fermentforschung
H.	Hoppe-Seylers Zeitschrift für Physiologische Chemie
Helv.	Helvetica chimica acta
Hofm. Beitr.	Beiträge zur chemischen Physiologie (Hofmeisters Beiträge)
Jahrb. wiss. Botanik	Jahrbuch für wissenschaftliche Botanik
J. Physiol.	Journal of Physiology
J. pr.	Journal für praktische Chemie
J. Biol. Chem.	Journal of Biological Chemistry
J. de l'Anat. Physiol.	Journal de l'Anatomie et Physiologie
J. de Pharm. Chim.	Journal de Pharmacie et de Chimie
J. gen. Physiol.	Journal of General Physiology
Klin. Wo.	Klinische Wochenschrift
Kolloidchem. Beih.	Kolloidchemische Beihefte
Kolloidz.	Kolloid-Zeitschrift

Münchener med. Wo.	Münchener medizinische Wochenschrift
Naturw.	Die Naturwissenschaften
Pflüg. Arch.	Archiv. für die gesamte Physiologie (Pflügers Archiv.)
Pogg. Ann.	Annalen der Physik (Poggendorffs Annalen)
Proc. Am. Soc. Biol. Chem.	Proceedings of the American Society of Biological Chemists
Proc. Chem. Soc.	Proceedings of the Chemical Society of London
Proc. Roy. Soc.	Proceedings of the Royal Society of London
R. Acc. Linc.	Atti della Reale Accademia dei Lincei (Rendiconti)
Rec. trav. chim.	Recueil des travaux chimiques des Pays-Bas
Skand. Arch. f. Physiol.	Skandinavisches Archiv für Physiologie
Soc.	Journal of the Chemical Society of London
Soc. Biol.	Comptes rendus de la Société Biologique
Virch. Arch.	Archiv. für pathologische Anatomie und Physiologie (Virchows Archiv)
Z. a. Ch.	Zeitschrift für anorganische und allgemeine Chemie
Z. Ang.	Zeitschrift für angewandte Chemie
Z. Biol.	Zeitschrift für Biologie
Z. El. Ch.	Zeitschrift für Elektrochemie
Z. f. ges. Brauw.	Zeitschrift für das gesamte Brauwesen
Z. exp. Med.	Zeitschrift für experimentelle Medizin
Z. f. klin. Med.	Zeitschrift für klinische Medizin
Z. rat. Med.	Zeitschrift für rationelle Medizin
Zbl. f. Bakt.	Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten
Zbl. f. Physiol.	Zentralblatt für Physiologie

ENZYME ACTIONS AND PROPERTIES

GENERAL SECTION

I. DEVELOPMENT OF THE FERMENT CONCEPT

"Fermentatio."—The knowledge and practical application of certain fermentative processes, especially alcoholic fermentation, has been general since prehistoric periods. The concept of the "fermentatio," however, which in ancient times embraced the most various phenomena and especially those processes accompanied by the evolution of gas, was still very indefinite and purely external in nature. It would be too much of a digression to attempt to discuss here the many variations of opinion with respect to the nature of all the various phenomena of fermentation which were known. These were, in fact, of little significance so long as the chemical principles of the ferment reactions were not definitely known.

Era of Liebig's Researches.—It was not until the period of Liebig's researches that more exact concepts were formed as to the course followed by fermentative processes. By that time, too, experimenters had become acquainted with a series of other enzyme reactions besides the fermentation processes, which had hitherto been all that were known. Kirchhoff [1] in 1814 had discovered the catalytic action of the glutinous component of wheat meal, the material being capable of converting starch to sugar and dextrin; Payen and Persoz [2] later separated an active preparation of this enzyme, amylase, from malt. The cleavage of amygdalin by the emulsin of bitter almonds was noted by Liebig and Woehler.[3] The protein splitting ferments had also been

[1] Schweigger's Journal für Chemie und Physik, **14**, 389 (1815).
[2] A. ch. **53**, 73 (1833); **56**, 337 (1834).
[3] A. **22**, 1 (1837); Pogg. Ann. **41**, 345 (1837).

discovered, Schwann [1] having described pepsin and Corvisart,[2] trypsin.

The decomposition theory of Liebig,[3] which postulated the ferment process as an alteration of the molecular equilibrium of the substrate following the chemical disintegration of the ferment itself, pictured these reactions for the first time under a unified concept. However, Liebig's mode of explanation could not long be maintained and was soon supplanted by the concept of Pasteur.[4]

Vitalistic Assumption of Pasteur.—Through Pasteur's classic investigations, the close connection between many ferment processes, such as alcoholic fermentation, and the vital activity of certain lower organisms was discovered. Such processes as those of fermentation and putrefaction, Pasteur ascribed merely to the metabolism of microorganisms. He considered that these processes could take place only within the living cell; in accordance with this, alcoholic fermentation was conceived as the life of yeast in the absence of oxygen. These concepts led investigators to make a distinction between these *formed* ferments, whose function was connected with the life of microorganisms, and those ferments which were observed to operate outside the cell, such for example as those of the digestive secretions of animals. Hence the latter were termed *unformed* ferments or *enzymes*.

The vitalistic concept of Pasteur and correspondingly the distinction between formed ferments and enzymes was accepted until the beginning of the present century. It could not be overthrown until it was demonstrated that this connection with the cell was variable in many cases, e.g., in the case of the cane-sugar splitting ferment of yeast. Observations which might have removed the distinction between formed and unformed ferments long remained unrecognized; in particular might be mentioned the observations made by Berthelot,[5] Traube,[6] and Hoppe-Seyler,[7] wherein it was noted that effective enzymes were likewise detectable within the living cell.

[1] Müllers Archiv 1836, p. 90.
[2] Zs. rat. Med. **7**, 119 (1858).
[3] A. **41**, 357 (1842); **153**, 1137 (1870); J. pr. N. F. **1**, 35, 312 (1870).
[4] Die Alkoholgärung, 2. Aufl., Stuttgart, 1878.
[5] C. r. **51**, 980 (1856).
[6] Ges. Abhandl., Berlin, 1899.
[7] Pflüg. Arch. **12**, 1 (1876).

To Buchner [1] belongs credit for the great service of having reestablished the unity of the ferment concept and breaking the more or less mystical spell of the vitalistic theory. He demonstrated that even the most important example of the formed ferments, namely, that of alcoholic fermentation, can under suitable conditions be separated from the living cell, that its action can be shown to exist in the expressed juices of yeast, independently of the presence of the cells themselves. The proof of this had hitherto been prevented merely by the instability of the ferment, since under the ordinary conditions which occasion destruction of the cell the ferment is likewise destroyed. In similar manner the separation of other ferments, such as those of lactic and acetic acids, was subsequently accomplished.

Definition of Enzymes.—The modern development of the doctrine of fermentation, namely, the identity of the concepts of "ferment" and "enzyme," begins with Buchner. Investigators were then enabled to continue their studies with respect to the nature and operation of ferments from a new and simplified point of view. The manner in which enzymes act, to be discussed more extensively in later chapters, may now be regarded as being clearly defined along broad lines. To the study of the course taken by enzymic processes, we owe recognition of the fact that the agents of these reactions, the enzymes, must be regarded as pure catalyzers. This idea had already been stated by Mitscherlich [2] and by Berzelius.[3]

Enzymes may be defined, therefore, as definite material catalyzers of organic nature with specific powers of reaction, formed indeed by living cells, but independent of the presence of the latter in their operation. Their action as catalyzers, therefore, consists only in the acceleration of reactions already in progress without themselves being consumed or altered by the reaction.

Material Nature of Enzymes.—Although, at the present time, differences of opinion as to the mode of enzymic action exist only with respect to special details, our foundations for another important phase of the above definition are much less assured, namely, with regard to the assumption of a definite material nature of these

[1] B. 30, 117 (1897); E. Buchner, H. Buchner and M. Hahn, Die Zymasegärung, Munchen, 1903.

[2] Pogg. Ann. **31**, 273 (1834).

[3] Lehrbuch der Chemie **6**, 20 (1837).

catalyzers. No success has been attained as yet by the Willstätter school in positively identifying any of the known enzymes as a chemical individual, in spite of the efforts at purification—in many cases quite extensive. In none of the cases investigated has it been possible to classify an enzyme as belonging to a definite group of chemical substances. It has likewise been demonstrated, in the case of many enzymes, that they can belong to none of the known classes of substances. All the views of both earlier and later writers in the literature of the subject with respect to the special chemical nature of enzymic substances have as yet proved speculative, so that today we possess no positive characteristic mark of these bodies beyond their specific mode of action. As a result of such experiences, it is easy to comprehend why there have been various objections raised, especially of late, to the concept of a definite material structure of these bodies, although the alternative concepts offered in their place correspond far less to the experiences of preparative enzyme chemistry.

Thus Fodor [1] has expressed and vigorously supported the view that enzymes are not to be regarded as special chemical individuals of peculiar chemical structure, but as known constituents of protoplasm, merely having a special degree of colloidal dispersity. According to Fodor an essential condition for exhibition of enzyme action is the maintenance of a definite degree of division, the loss of which occasions also the loss of enzymic properties. As a special application of this view we are o regard the cane-sugar, splitting enzyme of yeast as identical with yeast gum, the protein splitting enzyme of this organism with a phosphorus-containing constituent of protoplasm, the yeast-phosphorprotein. These are identical therefore with known elements of yeast, whose activities, according to this view, are due merely to a definite colloidal condition.

More pronounced yet is the contrast between the material concept of enzymes and the belief in their chemical individuality based on the investigations of Baur,[2] of Herzfeld,[3] and Ehrenberg,[4] according to which the action of enzymes is to be attributed rather to a suitable mixture of chemically familiar substances.

[1] Das Fermentproblem. Dresden und Leipzig, 1922.
[2] Bio. Z. **117**, 96 (1921); G. Schlatter, ibid. **131**, 362 (1922).
[3] Bio. Z. **64**, 103 (1914); **68**, 402 (1915); **88**, 260 (1918).
[4] Bio. Z. **128**, 431 (1922).

In this connection the authors refer to an artificial preparation of ferments, as in lactic acid fermentation by peptone, or in the hydrolysis of proteins by means of a mixture of albumen degradation products.

These concepts, however, have found no support in the preparative studies of Willstätter.[1] For one thing it was shown that, in the special case of yeast saccharase, it was possible to separate the enzyme completely and without loss of activity from the yeast gum with which Fodor had identified it. Also, it has been demonstrated in general that the dependence of this and other enzymes upon dispersity and division is not so significant as would appear from the purely colloid chemical point of view. Thus the effectiveness of the enzyme is not influenced at all even by pronounced dispersoid chemical alterations, as for example by adsorption on inorganic substances. If we consider moreover the results obtained by means of preparative investigations with respect to the specific adsorption behavior of the enzymes and to the smooth and facile separation from enzymic substances likewise closely related as to their specificity, such as the proteolytic enzymes of the pancreas,[2] merely upon the basis of specific adsorption affinities, we can have no further doubt that the enzymes do not react as more or less specific constituents of protoplasm (but with peculiar dispersity), but rather as definite and distinguishable chemical individuals. The highly definite structural specificity of these substances, likewise, can be suitably explained only in this way. It may be added that in the case of many important enzymes, preparative purification has shown, as already mentioned, that they cannot be identified chemically with any of the known constituents of protoplasm, and the special concepts concerning the synthesis of ferments from mixtures as published by Baur, Herzfeld and Schlatter can be refuted as erroneous.[3]

Still the collected observations made in preparative purification with respect to the stability of the enzymes, especially in a pure form, have led to the conclusion that even in the simpler cases, as in that of yeast saccharase, there is a certain dependence of enzymic activity upon the colloid state, and that the alterations

[1] B. **55**, 3601 (1922).

[2] Compare E. Waldschmidt-Leitz and A. Harteneck, H. **147**, 286 (1925).

[3] Chr. Barthel and H. v. Euler, H. **128**, 257 (1923); O. Acklin, Bio. Z. **139**, 457; **141**, 70; **142**, 117, 351 (1923).

of enzymic affinity cannot be explained merely as an influencing of the specific chemical group of the enzyme molecule. Willstätter [1] considers therefore that *the enzymes are composed of a colloidal bearer and a specific, active group, which enables them to be bound to the substrate, and the composition of which at the same time conditions the colloidal nature of the entire complex.* Such a concept gives equal value to the physico-chemical and to the structural chemical behavior of the enzymes. The problem of determining the specific material bearers of enzymic action suffers so much the less a loss in justification and significance, since it will be called on sooner or later to render a final decision between the various viewpoints as to the nature of enzymes.

[1] Compare R. Willstätter, J. Graser and R. Kuhn, H. **123**, 1, 45, 59 (1922).

II. ENZYMES AS COLLOIDS

Colloidal Characteristics.—The colloidal character of the enzymes and their solutions has already been discussed in the preceding chapter. Also, the somewhat too extensive conclusions which have been drawn with respect to the significance of these properties were treated in that chapter. As a matter of fact, all the enzyme preparations thus far described in the literature of the subject exhibit the distinctive characteristics of colloidal dispersion, even when taking into consideration the fact that these characteristics may be due to an admixture of high-molecular substances which were contained in the cell. Thus, even the most exhaustively purified solutions of yeast saccharase, in the experiments of Willstätter and Euler, exhibit the Tyndall effect which is characteristic of colloidal solutions. Further evidence that enzymes may be classed among colloids, in the usual sense of the term, is provided by their behavior towards semi-permeable membranes such as parchment, collodion, or animal membranes, which have been demonstrated as being generally impermeable to enzymes. By no means the least significant characteristic in this respect is their pronounced adsorption affinities, which will be discussed in detail in the following sections.

Colloidal characteristics appear to be most strongly marked in the water-insoluble, true intra-cellular enzymes, whose most characteristic example, according to Willstätter and Waldschmidt-Leitz,[1] is the fat-cleaving ferment of the ricinus seed. This lipase moreover is highly unstable with regard to water and electrolyte solutions; "it is attached either by adsorption to an insoluble bearer belonging to the protein group . . . or else the protein substance is the colloidal bearer of the lipatic active group as a constituent of the lipase molecule itself."

Particle Size.—The principal and distinguishing characteristic of colloidal state is given by the particle size, i.e., the extent to

[1] H. **134**, 131 (1922–23).

which the molecule of a substance is bonded into higher aggregates; according to the limits of colloidal substances this particle size varies between the diameters of 1 to 100$\mu\mu$. A number of the methods employed for determining this magnitude, such as ultra-filtration or the determination of freezing point lowering and boiling point elevations, are not applicable to the measurement of enzyme solutions, because of the disturbing factors caused by the presence of concomitant materials or their disintegration products. On the other hand, methods for molecular weight determinations based on the rapidity of diffusion have furnished certain avenues of attack for determining the magnitude of ferment particles. Such methods have the advantage of enabling the investigator to examine the enzymes independently of the substances found together with them in solution, in so far as there do not occur combinations of the enzyme with the accompanying substances, a point which must be taken into consideration. The velocity with which the enzyme diffuses from its solution toward the pure solvent is measured; the diffusion coefficient [1] calculated therefrom bears to the molecular weight M the simple relation:

$$D\sqrt{M} = \text{constant}$$

in which, according to Oeholm [2] the constant K is equal to 7.0 for water at 20°.

Diffusion Velocities and Molecular Weights.—The measurements of the molecular weights, which have been carried out according to this procedure by Herzog [3] with a series of enzyme solutions, and by Euler [4] with yeast saccharase, are given in Tables I and II.

As the tables indicate, the values obtained for the molecular weights vary between 10,000 and 45,000 while the value obtained by the same procedure with egg albumen amounts to 14,000. From the measurements by Euler with saccharase it is further obvious that from a certain degree of purity on, the diffusion capacities are fairly constant, and that consequently there is no

[1] Compare W. Kawalki, Wied. Ann. **52**, 166 and also p. 185 (1894); A. Einstein, Ann. d. Phys. **19**, 303 (1906); Z. El. Ch. **14**, 237 (1908).

[2] Ph. Ch. **50**, 309 (1904–05); **70**, 378 (1910).

[3] Z. El. Ch. **13**, 533 (1907); Bio. Z. **11**, 172 (1908).

[4] H. **73**, 335 (1911); **110**, 190 (1920); **113**, 59 (1920); Kolloidz. **31**, 3 (1922).

further perceptible influence occasioned by concomitant materials. It is to be noted, however, that, aside from the non-determinable influence of an association of the enzyme with accompanying substances, the figures here recorded represent only the lowest values of the molecular weight, since the Oeholm constant $D\sqrt{M} = 7$, based on results obtained with proteins, gives values which are too low. Nevertheless, these measurements show that the diameter of the enzyme particle corresponds in order of magnitude with that for the higher proteins.

TABLE I

Enzyme	D (18°)	M	Average Diameter r ($\mu\mu$)
Pepsin	0.070	10,000	4.2
Lab	0.066	11,200	4.4
Emulsin	0.036	37,700	8.2
Invertase	0.033	44,900	9.0

TABLE II

(Saccharase)

Degree of Purity (Inversion power)	(D (20°)	M
4.6– 3.8	0.0425	28,000
11.5– 7.7	0.047	22,000
84.0– 32.2	0.0488	20,600
220.0-200.0	0.0500	19,600

III. ENZYMES AS ELECTROLYTES

Significance of Hydrogen-Ion Concentration.—The idea that the effectiveness of enzymes depends upon their electric charge was first promulgated in 1909 by Loeb.[1] This agreed with the well established observation as to the dependence of the action of pepsin on the presence of strong acids. This dependence of enzyme action upon the acidity or alkalinity of the solution, which had been observed also in the case of other enzymes, failed for a long time to receive proper attention; a determination of titration acidities was usually considered sufficient for the measurement of enzyme preparations. Credit must be given to Sörensen[2] and Michaelis[3] for their recognition and emphasis of the fundamental importance of hydrogen- and hydroxyl-ion concentrations with respect to the activity of enzymes. The maintenance of a constant hydrogen-ion concentration, as introduced by these investigators for the measurement of enzyme preparations, tended to lessen the uncertainties with which many of these measurements had been hampered and to facilitate their ease of comparison. The importance which the consideration of H-ion concentrations has attained with respect to the development of enzyme chemistry justifies a brief description here of the procedures employed in the production and measurement of definite acidities.

Buffers.—As regulators for the production and maintenance of definite H-ion concentrations the so-called "buffers" recommended by Sörensen and Michaelis are used. These are suitable salt mixtures, whose acidity suffers no appreciable alteration, within certain limits, by the addition of acids, alkalies or amphoteric substances, and which are capable therefore of preventing an alteration of acidity due to products already present or products formed in the course of enzyme action. Especially fitted for this are salts of tribasic acids such as phosphoric or citric acid, and

[1] Bio. Z. **19**, 534 (1909).

[2] Bio. Z. **21**, 131, 255 (1909); Ergebn. d. Physiol. **12**, 393 (1912).

[3] See "Hydrogen Ion Concentration," Part I, Baltimore, 1926.

quite generally the salts of weak acids or bases or even amphoteric substances themselves. Table III which follows indicates a number of such buffer solutions together with their special region of value. The concentration of hydrogen-ions is expressed here by the usual simplified measure (proposed by Sörenson), that is, *p*H = hydrogen-ion exponent or hydrogen exponent, which represents the negative decimal logarithm of the H-ion concentration.[1]

TABLE III

BUFFER MIXTURES OF SÖRENSON AND MICHAELIS

Buffer Mixture	Region of Value (18°)	
	(H^+)	*p*H
Glycocoll—Hydrochloric acid....	$7.08\times10^{-2}-2.09\times10^{-4}$	1.15 – 3.68
Acetic acid—Acetate.....	$5.76\times10^{-4}-0.28\times10^{-6}$	3.24 – 6.55
Primary—Secondary phosphate.	$0.64\times10^{-5}-0.61\times10^{-8}$	5.19 – 8.21
Ammonia—Ammonium chloride.	$1.02\times10^{-8}-1.0\times10^{-11}$	7.99 – 11.00
Glycocoll—Caustic soda.....	$2.63\times10^{-9}-1.07\times10^{-13}$	8.58 – 12.97

Measurement of the H-ion concentration or *p*H of a solution is carried out according to the proposal of Michaelis, either by direct determination of the difference of potential against a hydrogen electrode, or more quickly, if somewhat less certainly, by colorimetric comparison with given indicators and a solution of known *p*H.

Activity—pH Curves.—By means of these methods there have been measured, first by Sörensen and by Michaelis, and later by other investigators, the dependence of the activity upon *p*H for a great number of enzymes; in this way a characteristic optimal region of action was determined for each of the enzyme preparations examined. The ratios obtained were commonly reproduced by the so-called activity-*p*H curves, which compare relative

[1] More precisely it may be said that *p*H represents the H-ion activity, since the effectiveness or activity of hydrogen ions is subject to some variations even for the same hydrogen ion concentrations. These variations are influenced by the nature of the buffer acid and the neutral salt, as well as the concentration of the neutral salt. See Michaelis and Perlzweig, "Hydrogen Ion Concentration," 1926, p. 127.

activity with the *p*H. Thus, for example, Michaelis and Davidsohn [1] obtained the *p*H curve reproduced here for the activity of the cane-sugar splitting enzyme of yeast, invertase. As is seen, the curve exhibits a broad optimal zone of action for the enzyme between *p*H 3.5 and 5.5.

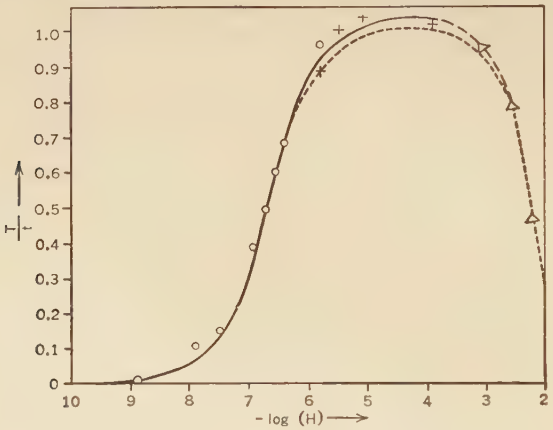


FIG. 1.—Activity-*p*H curve for invertase.

In Table IV, which follows, the acidity optima obtained for the most important enzymes are presented, in so far as these are known.

The table has reference only to the most exact and reliable measurements to be found in the literature; furthermore, the data refer almost exclusively to the crude enzyme preparations.

Disturbance of pH Dependence.—In many cases sufficient care has not been taken to avoid disturbing factors and errors which affect or distort the results of the measurement. Thus it must be borne in mind that the stability of the enzyme varies in proportion to the acidity. In fact, for some enzymes, as with yeast saccharase for instance, the optimum of stability coincides approximately with the optimum of activity; whereas with certain others, e.g., the trypsin of pancreas in crude preparations, the point of maximum stability does not occur in the region of its reaction optimum. Obviously in such cases the form of the activity-*p*H curve and the position of its maximum will be distorted unless care is taken to obviate the influence of enzyme

[1] Bio. Z. **35**, 386 (1911).

destruction, as for example by the employment of lower experimental temperatures and briefer periods for the determination. Other disturbances which are not always taken into consideration concern an effect produced by the hydrogen- or hydroxyl-ions on the substrate itself, which in many cases, as in proteolytic measurements, makes a control experiment absolutely necessary. Finally, there are cases, among which is included the splitting of true fats by animal lipases, in which the exact measurement of the reaction optimum is prevented because of the impossibility of excluding the influence of the cleavage products which occasion a marked alteration in *p*H.

TABLE IV

H-ION OPTIMUM FOR SEVERAL ENZYMES

Enzyme	*p*H Optimum	Observer
Pancreas lipase	8	Davidsohn [1]
Gastric lipase	4–5	Davidsohn [1]
Ricinus lipase	4.7	Haley and Lyman [2]
Pepsin	1.5–1.6	Sörensen [3]
Trypsin	7.8–8.7	Waldschmidt-Leitz [4]
Erepsin	7.8	Waldschmidt-Leitz and Harteneck [5]
Urease	7.0	van Slyke and Zacharias [6]
Saccharase	4.2	Michaelis and Davidsohn [7]
Maltase	6.1–6.8	Michaelis and Rona [8]
β-Glucosidase	5	E. Fischer [9]
Pancreas amylase	6.7–7.0	Sherman, Thomas and Baldwin [10]
Malt amylase	4.6–5.2	Lüers and Wasmund [11]
Catalase	7	Sörensen [12]

[1] Bio. Z. **49**, 249 (1913).
[2] Am. Soc. **43**, 2664 (1921).
[3] Bio. Z. **21**, 131 (1909).
[4] H. **132**, 181 (1923–24).
[5] H. **147**, 286 (1925).
[6] Jl. Biol. Chem. **19**, 181 (1914).
[7] Bio. Z. **35**, 386 (1911).
[8] Bio. Z. **57**, 70; **58**, 148 (1913).
[9] H. **107**, 176 (1919).
[10] Am. Soc. **41**, 231 (1918–19).
[11] Fermentf. **5**, 169 (1920–21).
[12] Bio. Z. **21**, 131 (1909).

The dependence of enzyme action upon the hydrogen number, and particularly the position of the reaction optimum, were regarded in the researches of the Michaelis school as a characteristic mark of the individual enzymes. Thus the determination of the *p*H optimum is applied in many cases to the differentiation

of individual enzymes; for example, it is considered that with animal amylases there should be a distinction between various amylase-salt complexes according to the different acidity optima which result from the addition of various neutral salts.[1] Meanwhile the researches of Willstätter [2] have shown that just as with many enzymes, so also with animal lipases, the dependence upon the hydrogen number, as determined, may be altered or distorted by entirely different, complicated factors; the influence of the hydrogen number will be obscured through the activation and inhibition by concomitant substances and reaction products. Thus it has been found that the lipase of the stomach, which, as shown in Table IV, was distinguished from the pancreas lipase on the basis of its acid reaction optimum in the crude state, i.e. in the gastric juice itself, could, in a purer condition, be identified with the pancreatic enzyme with respect to this property. In other words, it is found in its natural secretion together with a substance which inhibits its operation in an alkaline reaction; when this substance is separated out the position of its reaction optimum is advanced by degrees in order to coincide finally with that of the pancreatic enzyme; accordingly, it shifts from the acid to the alkaline region. Table V, which illustrates this behavior with the lipase of the dog's stomach, is taken from an investigation conducted by Willstätter, Haurowitz and Memmen.[3]

TABLE V

H-ion Relations for the Gastric Lipase of Dogs

Enzyme Preparation	Optimum *p*H	Minimum *p*H
Lipase solution from mucosa	5.5–6.3	8.6
Acetic acid precipitate	5.5–6.3	8.6
Electro-dialysate	6.3–7.1	4.7
After purification with kaolin	7.1–7.9	4.7

Enzymes as Amphoteric Electrolytes.—The theoretical significance attached by Michaelis [4] to the activity-*p*H curves for

[1] See L. Michaelis and H. Pechstein, Bio. Z. **59**, 77 (1914).

[2] R. Willstätter, E. Waldschmidt-Leitz and Fr. Memmen, H. **125**, 93 and particularly p. 97 (1922–23).

[3] H. **140**, 203 (1924).

[4] L. Michaelis and H. Davidsohn, Bio. Z. **35**, 386 (1911).

enzymes, is based upon the idea that the enzymes themselves are to be regarded as amphoteric electrolytes, i.e., as substances having a character at once basic and acid, which, according to the *p*H, act in varying capacities as anions, kations or as undissociated materials. The activity-*p*H curve for saccharase given in Fig. 1 corresponds, for example, according to Michaelis, at least in its alkaline branch, to the form of a so-called dissociation residue curve, i.e., to the undissociated part of saccharase, which may be expressed as a function of the H-ion concentration by means of the following equation:

$$\rho = 1 - \gamma = 1 - \frac{K}{K + (H^{\cdot})} = \frac{H^{\cdot}}{K + (H^{\cdot})}.$$

Here ρ signifies the dissociation residue, γ the degree of dissociation, and K the dissociation constant of the saccharase acid. For the special case where $(H^{\cdot}) = K$ in the above reaction, ρ is equal to 0.5, this being the one H-ion concentration for which the activity-*p*H curve indicates half the maximal reaction velocity; thus, in the example given $K = 10^{-6.7}$ for saccharase.

These results, according to Michaelis, imply that the undissociated molecule of yeast saccharase is to be considered as the active portion and, moreover, at various H-ion concentrations, only a certain portion of the enzyme is present in an undissociated, and therefore catalytically active, form. That the form of the curve here represents the dissociation residue of an acid, and not, as is likewise possible, the dissociated portion of a base, is concluded by Michaelis, the conclusions being drawn from researches upon the adsorption behavior and the migration of the enzyme in the electrical field, which indicate its acid character. The activity-*p*H curves of the other enzymes studied by Michaelis are explained in a similar manner; thus in the analysis of trypsin it was found that its effective portion was represented by the ferment anions.

The original conclusions of Michaelis, according to which only the undissociated portions of saccharase occasion enzymic activity and according to which activity is ascribed solely to the electrochemical character of the enzymes, has been strongly questioned, meanwhile, by the investigations of Michaelis and Rothstein [1] and by Kuhn.[2] In these investigations it is demonstrated, con-

[1] Bio Z. **110**, 217 (1920). [2] Naturw. **11**, 732 (1923).

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

trary to the theoretical conclusion, that the activity-*p*H curve of the enzyme in varying cane-sugar concentrations does not change and that on the other hand the equilibrium between enzyme and sugar is found to be independent of the hydrogen number. These observations do not support the concept of the union of the enzyme and cane sugar as an ionic reaction. From the fact that the reaction velocity is varied by H-ion changes, despite the constancy of the enzyme portion bound on the substrate and its independence of H-ions, it is much rather to be assumed, according to Kuhn, that the H-ions influence the decomposition velocity of the enzyme-substrate combination.

Ionization of the Substrate.—Although, in these investigations, the policy has been to attribute the dependence of activity on the H-number chiefly to the electro-chemical character of the enzyme itself, Northrop [1] has recently advanced and experimentally treated the hypothesis that, in the special case of the proteolytic enzymes, the reaction velocity depends, not upon the dissociation of the enzymes, but upon that of the substrate. The latter view is supported by the observation that in a series of instances the activity-*p*H curves of pepsin and trypsin exhibit a certain coincidence with the dissociation curves of the substrate employed. According to Northrop, pepsin, the activity optimum of which corresponds to a strong acid reaction, is capable of reacting only with the albumen kations, and trypsin, which is optimal in an alkaline reaction, only with the protein anions. According to the researches of Willstätter and Grassmann [2] and of Hertzmann and Bradley [3] there is a possibility here of differentiating a third group of proteolytic enzymes which attack only the undissociated protein molecules, their *p*H optimum corresponding to the isoelectric point of the substrate. Meanwhile the cases investigated are not sufficiently numerous and not variously enough chosen to enable us to reach a definite conclusion with respect to the ideas advanced. According to researches by the present author the Northrop theory does not agree with the experimental facts in a number of examples which have been investigated. It is preferable in general to take into consideration not only the dissociation of the substrate but also that of the enzymes.

[1] Naturw. **11**, 713 (1923).
[2] H. **138**, 184 and particularly p. 198 (1924).
[3] Proc. Am. Soc. Biol. Chem. **18**, 19 (1923).

Migration in Electrical Fields.—The behavior of enzymes in an electrical field is applied by Michaelis to the characterization of their electro-chemical nature. As is well known, the electro-negative anions migrate to the anode under the action of the electric current and the electro-positive kations to the kathode, while the direction of migration of amphoteric electrolytes such as amino acids and proteins is determined by the acidity of the solution. In an alkaline medium these ampholytes move towards the anode, in an acid medium towards the kathode, while in the intermediate region, corresponding to their isoelectric reaction, no migration at all is observed.

Influence of Associated Materials.—The researches of the Michaelis school,[1] together with earlier observations by Bierry, Henri and Schaeffer [2] as well as by Iscovesco [3] have constituted a thorough study of the migratory behavior of a series of crude enzyme preparations. It was demonstrated for example that, even in a strong acid solution, yeast saccharase exhibits only an anodic migration, while in the case of other enzymes such as malt amylase, trypsin and catalase it was shown that the direction of migration depends on the acidity. Michaelis, accordingly, ascribed to saccharase a purely or at any rate a predominatingly acid character. The subsequent inspection of these observations by Willstätter, Graser and Kuhn [4] with purer enzyme preparations has led to the conclusion, however, that they are not to be referred to the behavior of the enzyme itself. With an increase in the degree of purity, as is indicated in Table VI, there is a steady diminution in the electro-negative character of the saccharase. Hence " the electric migration, in so far as it is carried out with very impure enzyme solutions, reveals little as to the nature of a ferment but merely permits a decision as to whether the acid or the basic nature predominates in the addition or adsorption product formed by the ferment and its concomitant materials."[5] Accordingly, the nature of the electric charge is thus far not known with certainty either with saccharase itself or with the other enzymes.

[1] Bio. Z. **16**, 81; **17**, 231 (1909); **25**, 1 (1910); **30**, 481 (1911); **57**, 70; **53**, 320 (1913); **59**, 100; **64**, 13 (1914).

[2] Soc. Biol. **1**, 226 (1907); V. Henri, Bio. Z. **16**, 473 (1909).

[3] Soc. Biol. **1**, 770, 861 (1907); Bio. Z. **24**, 53 (1909–10).

[4] H. **123**, 1 and particularly p. 73 (1922).

[5] R. Willstätter, B. **55**, 3601 and particularly p. 3614 (1922).

TABLE VI

MIGRATION AND DEGREE OF PURITY OF SACCHARASE

Enzyme Preparation	Degree of Purity Time Value	Experimental Time Hours	Per Cent. Migration	
			Anodic	Kathodic
Yeast autolysate...	200	18	0.64	0.00
After adsorption on alumina.....	8.45	23	0.61	0.18
After adsorption on alumina and kaolin.....	1.0	22	0.54	1.93

Electro-chemical Adsorption.—Michaelis[1] also first analyzed the adsorption affinities of the enzymes in order to explain their electro-chemical nature. Adsorption is understood to be the enrichment of dissolved materials on the surface of solid bodies; it is differentiated from absorption, by which a solution enters into the absorbing material itself. The phenomena of adsorption, formerly attributed in part to capillary forces, i.e., to a decrease of surface tension at the interface of the phases[2] are being more and more explained from chemical viewpoints. Numerous experiments have been carried out to attribute adsorption phenomena to other influences than capillarity, for example, chemical action and electrical forces, and in many cases these indications have been conclusive. To Michaelis belongs the credit for having emphasized the chemical nature of many adsorption processes; his investigations, which in general discriminate between mechanical and electro-chemical adsorption, lead to the conclusion that the former is to be regarded as operative only in special cases, as for example in adsorption by charcoal or cellulose.

The phenomena of electro-chemical adsorption, which have been thoroughly investigated, for instance, in the adsorption of organic dyestuffs, are described according to the following viewpoints by Michaelis and Rona:[3]

[1] Bio. Z. **7**, 488 (1907–08); L. Michaelis and M. Ehrenreich, Bio. Z. **10**, 283 (1908); **25**, 359 (1910).

[2] Compare H. Bechhold, Colloids in Biology and Medicine, 2nd Ed. New York, 1919, p. 25.

[3] Bio. Z. **97**, 57 (1919); **102**, 268 (1920).

1. Equivalent adsorption—Taking up of the anions and kations of the salt in equivalent quantities;

2. Hydrolytic Adsorption—Taking up only separate types of ions;

3. Exchange Adsorption—Interchange between an ion of the adsorbent and one of the dissolved material; and

4. Discharge Adsorption—Taking up of the anions and kations in different proportions, accompanied by a change of electrical charge in the adsorbent.

Whereas, with insoluble, salt-like adsorbents exchange adsorption alone is noted, it is also true that adsorption by oxides of a basic or acid nature corresponds fully with a chemical transformation. "In the case of electrolyte adsorbents the adsorption is identical therefore with those reactions which can be predicted on the basis of ordinary chemical affinities." This observation corresponds with the theories of Langmuir [1] according to which adsorption results from the saturation of the free, externally directed, chemical valences at the interface of the adsorbent. The electrochemical character of the adsorption process permits a division, according to their electric charge, into electropositive and electronegative adsorbents, whose affinity for enzymic substances has led to significant conclusions concerning the electrochemical nature of the latter.

Adsorption Behavior of Enzymes.—The earliest observations regarding the adsorption of an enzyme, namely, pepsin, are found in the work of Brücke,[2] who had also already described a process for the liberation of the enzyme from the adsorbates, as likewise in the investigations of Danilewsky [3] and by Cohnheim [4] in the same decade. However, almost fifty years elapsed before Michaelis and Ehrenreich again took up the phenomenon of enzyme adsorption and proceeded to extend its application. By means of the guiding principle that all substances which are adsorbed by the electronegative adsorbent kaolin are to be regarded as bases, and that all those adsorbed by the electropositive alumina are to be regarded as acid, they investigated the chemical nature of a number of enzymes. The methods were not quantitative

[1] Am. Soc. **38**, 2221 (1916); **39**, 1848 (1917).
[2] Sitzungsber, Akad. d. Wiss. Wien **43**, 601 (1861).
[3] Virchow's Arch. **25**, 279 (1862).
[4] Ibid, **28**, 241 (1863).

but merely a sort of approximation. Invertase, which is adsorbed by alumina in every case, but on the contrary not in the slightest adsorbed by kaolin, exhibited according to this method the character of an acid, which is in full agreement with its migration behavior. Saliva amylase and trypsin, which are taken up by alumina as well as by kaolin in every reaction, were regarded on the other hand as amphoteric substances.

These observations, which were accentuated in crude enzyme solutions, do not hold good for the enzymes themselves as proven by Willstätter,[1] but only for their aggregates with concomitant substances. Thus it has been demonstrated that invertase is adsorbed from purer solutions by kaolin, and also even from crude yeast autolysates under certain conditions; hence it possesses basic as well as acid properties. Trypsin, after an imperfect separation, is no longer taken up at all by alumina, but, on the other hand, is taken up quantitatively by kaolin; its basic nature is thus demonstrated. Finally, animal amylase is less and less adsorbed by both basic and acid substances as its degree of purity increases. Hence the true adsorption behavior of enzymes is only recognizable in the higher degrees of purity.

Preparative Adsorption Methods.—The development and systematic application of adsorption methods has attained decisive significance in preparative enzyme chemistry through the numerous investigations by Willstätter and his co-workers. These methods have served not only to increase the degree of purity of many enzymes and other high molecular substances, but has also made it possible in many cases to separate enzymes from each other in their natural mixtures and to analyze enzymic reaction systems into their various components. The special adsorption processes carried out in these investigations will be illustrated by characteristic examples in Chapter VII. Their general directions and theoretical principles and results are treated in the immediately following paragraphs.

According to the results of actual experience, adsorption from enzyme solutions is not confined only to the enzymes themselves; rather, varying quantities of other high molecular concomitant materials are taken up by the adsorbent together with the enzymes.

[1] R. Willstätter and F. Racke, A. **425**, 57 (1920–21); R. Willstätter and W. Wassermann, H. **123**, 181 (1922); R. Willstätter and E. Waldschmidt-Leitz, H. **125**, 132 and specifically p. 139 (1922–23).

The success obtained in the preparative purification of enzymes by adsorption depends, therefore, according to Willstätter,[1] upon whether one succeeds in making the process of adsorption sufficiently discriminative. As a measure of the discriminative action of an adsorbent Willstätter and Wassermann[2] have introduced the term "adsorption value (*A. W.*)"; it is equal to the number of enzyme units which will be taken up by one gram of the adsorbent under the conditions of the experiment. The higher the A. W. in an experiment, the greater increase in enzyme concentration is to be expected under the given conditions. We find that, aside from the acidity of the solution, the adsorption value is in great measure dependent on the initial concentration of the enzyme and likewise upon the different enzymes in another way. It has been found by the researches of Willstätter and Wassermann and those of Kraut and Wenzel[3] that the quantity of yeast invertase taken up by the adsorbent alumina increases considerably in proportion to increasing dilution; thus in one instance the percentage of the enzyme portion absorbed by a given quantity of alumina rose upon a 50-fold dilution from 19 to 93 per cent. This phenomenon is attributed to an extensive dissociation at the greater dilution of one of the adsorption-disturbing association products from the enzyme and accompanying materials. On the other hand, in the case of animal lipase,[4] the adsorption behavior of which is less specific, no influence of the initial concentration has been definitely recognized, and vegetable peroxydase[5] appears to be even less adsorbed by alumina from more dilute solutions.

Adsorption Curves.—The influence of dilution upon the adsorption of invertase by alumina is represented by Kraut and Wenzel by the diagram in Fig. 2 which graphically illustrates the dependence of the adsorption value upon the concentration of the enzyme in the solution of the adsorption residue.

According to Kraut and Wenzel, the form of the adsorption curves obtained for a given enzyme preparation and their depend-

[1] B. **55**, 3601, explicitly p. 3610 (1922).

[2] H. **123**, 181 (1922).

[3] H. **133**, 1 (1923–24); **142**, 71 (1924–1925).

[4] R. Willstätter and E. Waldschmidt-Leitz, H. **125**, 132, explicitly p. 165 (1922–23).

[5] R. Willstätter and A. Pollinger, A. **430**, 269 and particularly 276 (1922–23).

ence on the initial concentration of the enzymes in the solution permits the recognition as to whether the enzyme is accompanied by the mechanical admixture of concomitant materials, which partially engage the surface of the adsorbent. The form of the adsorption curves in accordance with the relation proposed by Freundlich [1]

$$A.V. = \alpha \cdot c^{1/n}$$

(c = concentration in the residual solution, α and n = constants) for the adsorption behavior of pure material should correspond to the logarithmic form. It is of great importance for the selection of the most fitting preparative process that a separation of the concomitant materials should be secured, either by their previous adsorption or by a more selective adsorption of the enzymes themselves. The analysis and valuation of the characteristic adsorption curves for an enzyme solution, however, do not permit the differentiation and separation of the enzyme from such admixtures as are closely related to it in adsorption behavior, for example, its decomposition products. There are cases furthermore in which the adsorption of the enzyme is due to association with some admixture, as in the case of pancreatic amylase,[2] in which the adsorption curve therefore merely expresses the behavior of this complex. Nevertheless, in other cases the union of the enzyme with companionate substances, which are not absorbed, may succeed entirely in preventing its adsorption (compare the adsorption behavior of crude invertase against kaolin, according to Michaelis).

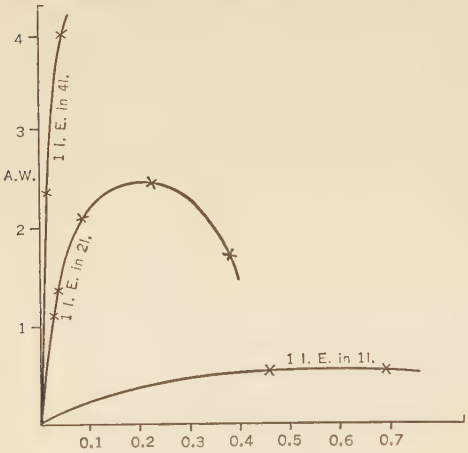
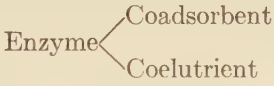


FIG. 2.—Adsorption value of alumina and initial concentration.

[1] Colloid and Capillary Chemistry, London, 1926, p. 172.
[2] H. **142**, 14 (1924–25).

Elution.—The influence of an association with concomitant substances upon the behavior of enzymes is particularly marked in the process of their liberation from adsorbates, i.e., elution, which, as Willstätter has shown, may be effected in general by an alteration of the reaction medium. For example, an enzyme adsorbed in an acid reaction is elutriated with dilute alkalis. On the other hand, elution appears in some cases to depend upon a pure displacement of the enzyme from the surface of the adsorbent, as in the use of phosphate as elutrient for alumina adsorbates through the formation of difficultly soluble aluminum phosphate; this is much more unspecific.

The numerous phenomena exhibited by the adsorption and elution behavior of the enzymes are explained, according to Willstätter and Racke,[1] by the theory that certain concomitant substances, "coadsorbents" and "coelutrients," are responsible for the ready adsorption or elution of the enzyme. According to this concept the complex



is both adsorbed and elutriated as well as the enzyme itself, while, on the other hand, the associated complex of the enzyme with a coadsorbent alone are held fast in the adsorbates.

Worth noting is the elutriating effect of substrates on enzyme adsorbates, which was first established by Hedin[2] for the adsorbates of trypsin on animal charcoal, and later observed also with carbohydrate splitting enzymes.[3] Moreover, the theoretical significance of this phenomenon is not to be disregarded.

[1] A. **425**, 1 and particularly p. 59 (1920–21).

[2] Biochem. Jl. **1**, 484 (1906); **2**, 81 (1907); H. **50**, 497 (1907); see also E. Waldschmidt-Leitz, A. Schäffner and W. Grassman, H. **156**, 68 (1926).

[3] R. Willstätter and R. Kuhn, H. **116**, 53 (1921); L. Michaelis, Bio. Z. **115**, 269 (1921).

IV. ENZYMIC KINETICS

Mass Action Law.—Those principles, deduced on the basis of chemical dynamics, which regard enzymic reactions as catalytic processes are by no means applicable to all enzyme reactions. The complicated and imperfectly understood composition of enzymic reaction systems, together with our ignorance of the chemical nature of the effective catalyzers and the influence of adventitious impurities upon their affinity, often results in preventing a classification of even the simpler transformations, along basic principles. When therefore in the older kinetic-reaction researches the application of the mass action law in one form or another has found expression through the establishment of mono-molecular, bi-molecular, or other reaction equations, the validity of such relations is in many cases of very limited value because of the disregard of numerous factors which determine the reaction. The object of this book, therefore, is to mention only the most typical examples which are best supported by experimental evidence.

Mono-molecular Reactions.—For the transformation of any single type of molecule into another, the law of mass action, according to which the active mass of a substance is proportional to its concentration, requires that the velocity of transformation should be found proportional to the concentration of the reacting molecules at any given time. This relation for the course of mono-molecular reactions is expressed by the equation

$$v = \frac{dx}{dt} = k(a - x)$$

in which a represents the initial concentration of the substance, x the amount of the substance already transformed in the given time t, and k a constant, the so-called mono-molecular reaction constant. By integration of this equation we obtain

$$k = \frac{1}{t} ln \frac{a}{a - x}$$

This relation for the course of mono-molecular reactions is valid, according to the classical researches of Wilhelmy [1] as illustrated in Table VII, for the catalytic inversion of cane sugar by acids. The course of this reaction in pure water is immeasurably slow and, although it is actually a bi-molecular reaction, it approximates a mono-molecular reaction in dilute solutions because of the practically unchanged quantity of one of the reaction components, namely, the water. In this case the constant of the reaction velocity is found to be proportional to the concentration of the catalyzer, the hydrogen-ions, whereas it is found to be independent of the cane-sugar concentration; hence in a given unit of time equal fractions of the substrate are transformed.

TABLE VII

Reaction Course of Cane-Sugar Inversion by H-ions

Time (t) Minutes	Angle of Rotation, Deg.	$K=\frac{1}{t}\log_{10}\frac{a}{a-x}$
0	+46.75	
45	+38.25	0.001 34
120	+26.00	0.001 38
240	+11.50	0.001 40
450	− 4.50	0.001 47
630	−10.00	0.001 39
∞	−18.70	.....

The validity of the mono-molecular reaction equation holds likewise for certain simple enzymic reactions, in so far as precautions are taken that the reaction conditions remain unchanged through the duration of the experiment and that the quantity of active enzyme is not diminished by the destructive influences. This has been demonstrated, to cite only a few examples, by Euler [2] as well as Dernby [3] for the hydrolysis of simple peptides by erepsin or by Issajew [4] for the decomposition of hydrogen peroxide by yeast catalase, from which latter investigation Table VIII is taken.

[1] Pogg. Ann. **81**, 413, 499 (1850).
[2] H. **51**, 213 (1907).
[3] Bio. Z. **81**, 107 (1916–17).
[4] H. **42**, 102 (1904); **44**, 546 (1905).

TABLE VIII

REACTION COURSE FOR YEAST CATALASE

Time (t) Minutes	Cleavage Per Cent.	$10^4 \cdot K = \frac{1}{t} \log_{10} \frac{a}{a-x}$
5	24.6	245.7
10	43.3	246.4
15	57.2	245.5
20	67.7	245.4
25	75.6	245.0
30	81.9	246.9

Varying Kinetics of Saccharase Action.—Furthermore, the enzymic hydrolysis of cane sugar by yeast saccharase has been described by Hudson [1] as a mono-molecular reaction. This is contradicted, however, by the observations of other investigators, according to which the reaction constant of the first order plainly rises here; thus, Hudson's results are explained by Michaelis and Davidsohn,[2] who regard the latter case as the normal one, by assuming that the enzyme is partially destroyed during the process.

The increase of the reaction constants, as expressed in the researches of Michaelis and Davidsohn, is shown in Table IX. For this case, Michaelis and Menten,[3] following the example of Henri,[4] have theoretically deduced a reaction equation for enzymic cane-sugar hydrolysis, which is in agreement with the experimental results and according to which the reaction velocity is expressed by the sum of a linear and a logarithmic function.

The investigations of Willstätter, Graser and Kuhn[5] have shown, meanwhile, that the phenomena of varying kinetics, as they appear, for example, in the divergent observations of Hudson and of Michaelis and Davidsohn, are not to be explained by a variable stability of the saccharase. These phenomena are repeated with purer enzyme preparations and also under conditions wherein no

[1] Am. Soc. **30**, 1160 (1908).
[2] Bio. Z. **35**, 401 (1911).
[3] Bio. Z. **49**, 333 (1913).
[4] H. **39**, 194 (1902); C. r. **135**, 916 (1902).
[5] H. **123**, 1 and particularly p. 63 (1922).

destruction of the enzyme takes place, and moreover, the time course of cane-sugar hydrolysis is shown to be in great measure dependent upon the hydrogen number. According to these investigations, the exact mono-molecular process of Hudson as well as the example of Michaelis and Davidsohn become special cases, when the hydrogen number is varied; the underlying cause of these deviations is not yet known, however.

TABLE IX

REACTION COURSE OF SACCHARASE ACTION

(*According to Michaelis and Davidsohn*)

Time (t) Minutes	$K = \frac{1}{t} \log_{10} \frac{a}{a-x}$
21.0	0.001 45
60.0	0.001 51
130.0	0.001 64
190.2	0.001 71
246.0	0.001 76

Enzyme Quantity and Reaction Velocity.—The requirements of the mass action law, which are entirely fulfilled in the case of H-ion catalysis, namely, the proportionality between catalyzer-concentration and reaction velocity, are likewise fulfilled in certain enzymic catalyses. As shown by the fundamental investigations of O'Sullivan and Tompson,[1] of Hudson,[2] and of Euler and Svanberg,[3] this ratio, according to which the transformation is found proportional to the product of the enzyme quantity and the time of action, holds fairly well, e.g., in a region of enzyme quantities of the ratio of 1:8. This is made evident in Table X, taken from Hudson.

This table likewise shows that another rule which might be expected in a purely catalytic reaction does not appear to hold good here, namely, the non-dependence of the reaction velocity upon the concentration of the substrate. This deviation, which

[1] Soc. **57**, 834 and particularly p. 848 (1890).
[2] Am. Soc. **30**, 1564 and particularly p. 1575 (1908).
[3] H. **107**, 269, and particularly p. 275 (1919).

distinguishes enzymic from H-ion catalysis, and which has become of great moment in the study of the reaction mechanism of enzymic cane-sugar hydrolysis, is verified by a long series of earlier researches, and has been tested with particular thoroughness by Michaelis and Menten [1] and by Nelson and Vosburgh.[2] The theoretical conclusions which have been derived from this phenomenon will be treated still more explicitly.

TABLE X

SACCHARASE QUANTITY AND REACTION VELOCITY

(*According to Hudson*)

Relative Saccharase Concentration	Time, Minutes	Transformation (Per Cent) with Initial Concentration of Sugar of		
		4.55 Per Cent	9.09 Per Cent	27.3 Per Cent
2.00	15	73.2	45.3	11.2
1.50	20	73.2	44.8	11.2
1.00	30	72.9	45.3	11.5
0.50	60	72.9	45.2	11.4
0.25	120	73.1	45.2	10.9

The Schütz Rule.—We will discuss briefly at this point another basic relation which appears to be valid for the course of certain enzyme reactions. In 1885 Schütz [3] formulated the law that the quantities of protein hydrolyzed in equal periods of time by various quantities of pepsin are proportional to the square roots of the quantities of ferment, accordingly giving the equation:

$$x = k\sqrt{F}. \quad (F = \text{Ferment Quantity})$$

Further, with a constant amount of ferment, in some reactions, the transformation has been shown to be proportional to the square root of the time, that is,

$$x = k\sqrt{t}$$

[1] Bio. Z. **49**, 333 (1913).
[2] Am. Soc. **39**, 790 (1917).
[3] H. **9**, 577 (1885).

This leads to the conclusion, according to Arrhenius,[1] that the reaction velocity is not only proportional to the substrate concentration existing at the time, but is furthermore inversely proportional to the quantity already transformed. A simple example of the truth of this is furnished by ester saponification with small quantities of hydroxyl-ions, e.g., by ammonia, in so far as the substrate is present in great excess. The reaction product, in this case the NH_4 ions formed, exerts an inhibiting action according to its concentration upon the velocity of catalysis; expressed differently, the NH_4 ion represses the dissociation of the ammonium hydroxide.

TABLE XI

Reaction Course of Ethyl Acetate Hydrolysis by Ammonia

(*According to Arrhenius*)

Time (t)	x (Observed) Per Cent	x (Calculated) $= 17.3\sqrt{t}$ Per Cent
1	17.5	17.3
2	25.5	24.5
5	38.5	38.7
10	51.2	54.2
18	63.1	73.4
26	71.4	88.2

It is evident from Table XI that the agreement between theory and observation holds good, at least up to a transformation of 50 per cent, for the hydrolysis of ethyl acetate by ammonia.

Enzyme-Substrate Compounds.—The dependence of reaction velocity upon the substrate concentration, as described above in the example of cane-sugar cleavage by saccharase, and as also found in the action of other enzymes, and the recognition of the great influence exerted by the cleavage products on the course of enzymic reactions, has led to the conception of *an attachment between the enzyme and the substrate on the one hand and between the enzyme and the cleavage products on the other.* According to this the velocity of enzyme reactions is determined by the concentra-

[1] Medd. fran. K. Vetenskapsak. Nobelinst. **1**, Nr. 9 (1908).

tion of the enzyme-substrate compound; in other words, it is this alone which furnishes the molecules through which the reaction proceeds. Hence, we must regard as inadequate the earlier investigations which describe the reaction velocity as a measure of the enzyme quantities, without taking into consideration the influence of the substrate concentration and of the cleavage products. In 1913 Michaelis and Menten [1] published the results of fundamentally new researches specially devoted to the kinetics of enzyme cane-sugar hydrolysis, in which the conditions were so chosen that the disturbing influence of the reaction products was excluded; they determined the initial velocity of inversion with varying concentrations of cane sugar.

Substrate Concentration and Reaction Velocity.—These researches, which, in contrast to the earlier investigations, maintained a constant and optimal H-number and excluded the observed mutarotation of the glucose formed, led to the conclusion that the absolute amount of inverted sugar underwent a notable increase with an increase in concentration, attaining finally a maximal value at a definite degree of concentration. The latter concentration in the example given in Fig. 3 corresponds to a normality of the substrate of 0.192. This concentration is in close agreement with confirmatory results obtained subsequently by Nelson and Vosburgh.[2]

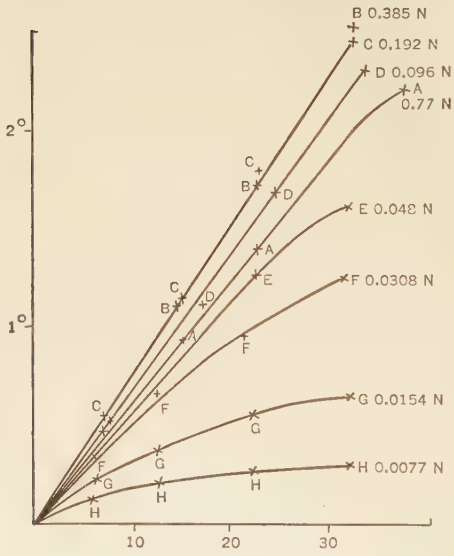


FIG. 3.—Reaction velocity and cane-sugar concentration.

(*According to Michaelis and Menten*)

Abscissae: Time in minutes. Ordinate: Decrease of rotation in degrees.

Further investigations of the relation between sucrose concentration and velocity of hydrolysis have shown that at con-

[1] Bio. Z. **49**, 333 (1913).

[2] Am. Soc. **39**, 790 (1917).

centrations considerably higher than 0.192 N, the rate of inversion drops steadily with increasing sucrose concentrations. An explanation of this phenomenon has been provided by the very careful kinetic measurements of Nelson and Schubert.[1] From a parallelism noted between the relative drop in velocity of inversion and the corresponding relative drop in water concentration, they conclude that the decrease in water concentration is the most important factor involved in the observed decrease in velocity (at concentrations above 6 per cent sucrose). This was demonstrated by experiments in which the sucrose concentration was maintained constant and the water concentration varied. That is, the water concentration was varied by adding such substances as glycerine, glucose and alcohol, which effect its displacement; by this means the sucrose and water concentrations are not dependent variables.

The important and noteworthy theoretical conclusions by which Michaelis and Menten arrived at an explanation of their experimental results obtained with dilute solutions will be treated more extensively in the following pages, since these principles appear to be indispensable to the comprehension of the newer concepts of the mechanism of enzymic reactions, and to the elucidation of many important factors which influence enzymic reaction velocity.

Michaelis and Menten proceed from the assumption that saccharase first forms a labile compound with cane sugar, which then breaks down into free enzyme and the cleavage products, glucose and fructose; they hold further that the velocity of cane-sugar inversion is conditioned by the existing concentration of the enzyme-cane-sugar compound, and is therefore proportional to the latter.

Application of the mass action law to the combination of the enzyme and cane sugar results, according to Michaelis and Menten, in the conclusion that the following equation is valid for the formation of a compound of 1 molecule of enzyme and 1 molecule of sugar:

$$\frac{(R)(\Phi - \phi)}{\phi} = K_M$$

In this R represents the total concentration of the cane sugar, Φ the total concentration of the enzyme, and ϕ the concentration of the combined enzyme, i.e., the enzyme-cane sugar combina-

[1] Am. Soc. **50,** 2188 (1928).

tion, while K_M designates a constant, and in fact, as is evident, designates the dissociation constant of the enzyme-cane sugar combination. The above equation may also be modified thus:

$$\phi = \Phi \cdot \frac{R}{R + K_M}$$

and since from the given assumption, the initial velocity v is proportional to the enzyme substrate compound, it may be expressed as

$$v = K \cdot \Phi \frac{(R)}{(R) + K_M}$$

or with the retained constant Φ in a series of experiments:

$$V = \frac{v}{K \cdot \Phi} \quad \text{and} \quad V = \frac{R}{R + K_M}$$

The expression obtained for V has a purely formal coincidence with that calculated for the dissociation residue of an acid, namely:

$$\rho = \frac{(H^{\cdot})}{(H^{\cdot}) + K}$$

(see Chapter III). Hence the graphic evaluation of the experimental results for the relations between the initial velocity and the negative logarithm of the initial concentration of the cane sugar must take the form of a dissociation residue curve. This requirement was satisfied in fact by all the observed relations in the research by Michaelis and Menten, so that the theoretical premises of their process obtained thereby a certain confirmation.

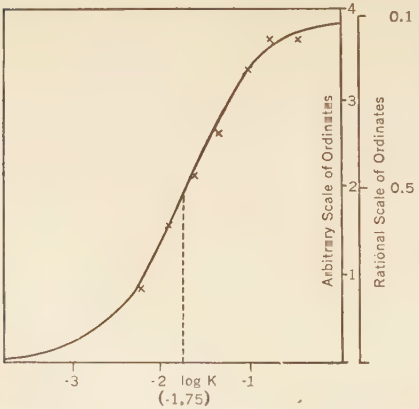


FIG. 4.—Activity-pS curve for saccharase. (*According to Michaelis and Menten*)

Activity-pS Curves.—The expression derived from the experimental results of Michaelis' researches are given in the form of a curve in Fig. 4. Such a curve is designated as an

"Activity-*p*S Curve" in order to emphasize its formal analogy to the activity-*p*H curve, since it represents the dependence of activity upon the substrate concentration (S). The activity-*p*S curve is considered analogous, in that the maximal possible reaction velocity is selected as the unit of the ordinate and in that the abscissa is based upon the negative logarithms of the substrate concentrations; it is not necessary here to consider more closely its determination, which is based upon the measurement of the angle of inclination of the middle, practically straight-lined portion of the curve.

Dissociation Constants.—From the activity *p*S curve we may derive, on the basis of the said relations as they have been developed for the activity-*p*H curve (Chapter III), the desired dissociation constants of the enzyme-substrate compound K_M, namely, in the equation

$$V = \frac{(R)}{(R) + K_M} \text{ for } K_M = (R)\ V = \tfrac{1}{2};$$

the dissociation constant K_M corresponds therefore in the curve to that substrate concentration for which there was found the half of the maximal initial velocity. Thus Michaelis and Menten, by this graphic process, have obtained the following figures for the dissociation constant of the saccharase-cane sugar compound. These figures are based on four series of experiments, carried out with various quantities of enzymes, and are in good agreement among themselves.

Relative Enzyme Quantity	$-\log K_M$	K_M
0.5	1.80	0.0160
1.0	1.78	0.0167
1.0	1.78	0.0167
2.0	1.78	0.0167

These results have made possible for the first time a conception as to the magnitude of the affinity existing between an enzyme and its substrate and have provided a numerical expression for

this affinity. Thus the value, 0.0167, obtained for the dissociation constant K_M, signifies that if it were possible to obtain the saccharase-cane sugar compound in pure form and to dissolve it in so much water that its undissociated portion was present in molar solution (that its molecular weight therefore was contained in one liter), then the solution besides would still exhibit $\sqrt{0.0167}$ or 0.133 moles of free saccharase and the same molar quantity of free cane sugar.

Affinity Variations.—The relation postulated and experimentally determined by Michaelis and Menten, according to which

$$K_M = \frac{\text{(Saccharase).}\quad\text{(Cane Sugar)}}{\text{(Saccharase—Cane Sugar Compound)}}$$

is found constant, was subsequently examined and verified in 1919 by Euler and Laurin [1] with a saccharase preparation from Swedish brewery yeast; it was confirmed once more that a satisfactory constant was obtained for the value of K_M. But the researches of these authors led to the surprising result that the absolute values of the dissociation constants varied quite considerably from those obtained previously by Michaelis and Menten; they found, for example, as the average value of their total experiments the much greater constant, $K_M = 0.026$.

The question naturally arising from this lack of accord, namely, whether there exists a definite and constant affinity between the enzyme and its substrate in the case of any given enzyme, has been studied along two lines by Kuhn.[2] This investigator has compared the affinities of saccharase from different races of yeast and also the affinities with varying degrees of enzyme purity. The experiments showed that the saccharase of a given yeast race suffered no appreciable alteration of its affinity to cane sugar during the course of purification, although such purification markedly influenced many of its other properties, e.g., its behavior towards adsorption media or electrical currents. On the other hand, very considerable variations of this magnitude were noted in the comparative study of saccharase from different yeast races; the constant K_M varied between 0.016 and 0.040 as is seen in Table XII.

[1] H. **108**, 64 (1919). [2] H. **125**, 28 (1922–23).

TABLE XII

AFFINITY OF YEAST SACCHARASE

(*According to Kuhn*)

Enzyme Preparation	Time Value, Minutes	K_M	Affinity Constant $= \frac{1}{K_M}$
Distillery yeast, Race 12, Autolysate	.....	0.016	63
Preparation from American brewery yeast	6.3	0.020	50
Lion brewery yeast, Munich, autolysate	180.0	0.029	35
Preparation therefrom	0.85	0.029	35
Preparation from another Lion brewery yeast	0.20	0.040	25

The observed phenomenon of varying affinity between an enzyme and its substrate, which is expressed in a varying dependence of its activity upon the substrate concentration, is of great influence, according to Kuhn, on the accuracy of the analytical determinations of the enzyme and on the applicability of the measure selected for the quantity and of the concentration, because with equal substrate concentrations here, under the conditions of the determination, temporarily unknown and varying fractions of the maximal reaction velocity can be given. This makes it necessary for the direct comparison of enzymes in various starting materials, to provide the values found with an index, which indicates their affinity or the dissociation constant of the enzyme substrate compound. Simple measurement of the reaction velocity is not sufficient in such cases.

Formation and Decomposition of the Enzyme-Substrate Compound.—According to Kuhn, the cause of such difference of affinity in various enzyme solutions is due to associations of the colloidal enzyme bearers with bodies which bear a close relation to the enzyme from a chemical viewpoint, although inactive in themselves, and resistant to boiling temperatures. The presence of these bodies exerts a marked influence on the activity of the specific group of the enzyme. It was found possible, for example, to reduce decidedly the affinity of a given saccharase solution by adding another solution of the enzyme which had been inactivated by boil-

ing. This reduction was to a constant, lower value, and the minimum was explained as being a saturation of the enzyme with the inhibiting substances. The above-mentioned basic principles of Michaelis, according to which the effect of an enzyme depends initially upon the formation of a labile compound of the enzyme with its substrate and next upon its decomposition into the free enzyme and into the reaction products, permit the assumption that the *reaction velocity* for an enzymic process, measured under any given set of conditions, is conditioned, on the one hand, *by the velocity of formation of the enzyme-substrate compound, which is expressed by the affinity constant of the enzyme to its substrate and, on the other hand, by the decomposition-velocity of the enzyme substrate compound.* It is to be expected correspondingly, that the velocity of an enzymic reaction will be influenced also by the second element of the described process, i.e., the decomposition tendency of the enzyme-substrate complex, in so far as this is not subordinate in degree of magnitude to the first element, i. e., the velocity of formation. A change in experimental conditions, whether expressed by an acceleration or by a retardation, will also become operative either with respect to the first process or to the second or to both. Hence in the following treatments which will take up certain special influences upon enzymic kinetics, particularly the carefully studied kinetics of cane-sugar hydrolysis, the question to which special attention should be given is to which partial process of the total reaction the separate influences are to be ascribed.

Influence of Reaction Products.—The fact that the velocity of many enzymic reactions is influenced by the presence of reaction products is in agreement with the demand made by the mass action law, according to which each substance concerned in the reaction determines the course of the reaction in proportion to its concentration. This phenomenon has long been recognized in the case of enzymic cleavages and has been a matter of frequent observation. Nevertheless, the researches of Michaelis and Menten [1] were the first which undertook to derive an exact, numerical expression for the influence of the cleavage products in the special case of the cane-sugar hydrolysis by saccharase. The experimental procedure with which these investigators ascertained the magnitude of the affinity of the enzyme to the cleavage products

[1] Bio. Z. **49**, 333 (1913).

of cane sugar consisted in the determination of the influence of added cleavage products upon the initial velocity of cane-sugar hydrolysis under like conditions.

The results of their measurements, which permit comparison as to the effect of the addition of glucose or fructose upon the velocity of cleavage, are summarized in Table XIII. These show that the initial velocity is clearly diminished by the presence of either reaction products.

TABLE XIII

INFLUENCE OF CLEAVAGE PRODUCTS ON CANE-SUGAR HYDROLYSIS

Time, Minutes	Rotation Change in Degrees		
	0.1 N Cane Sugar	0.1 N Cane Sugar 0.1 N Glucose	0.1 N Cane Sugar 0.1 N Fructose
0.5	0.011	0.010	0.009
30.0	0.703	0.610	0.554
46.0	0.935	0.855	0.785

Michaelis and Menten conclude from these findings that the concentration of the enzyme-cane sugar compound is diminished by addition of the cleavage products, since according to their premise the initial velocity is to be regarded as proportional to this concentration. In this case, accordingly, there occurs a distribution of the enzyme between the substrate and the cleavage products. The relation of the initial velocities measured corresponds then to the existing concentrations of the enzyme-substrate compound:

$$v : v_0 = \phi : \phi_0$$

wherein v_0 or ϕ_0 refer to the experiment made with cane sugar alone.

According to the mass action law, the following relations hold for the distribution of the enzyme on the substances, cane sugar and fructose, for example:

$$(R) \cdot (\Phi - \phi - \psi) = K_M \cdot \phi$$

and

$$(F) \cdot (\Phi - \phi - \psi) = K_{M_1} \cdot \psi$$

wherein Φ indicates, as before, the total concentration of the enzyme and ψ the concentration of the enzyme-fructose compound. For the dissociation constant of the enzyme-fructose compound K_{M_1}, we obtain from these two equations, by elimination of ψ:

$$K_{M_1} = \frac{(F) \cdot K_M}{(R) \cdot \left(\frac{\Phi}{\phi} - 1\right) - K_M};$$

by means of the relation $\phi = \frac{v}{v_0} \cdot \phi_0$ and the one given previously, according to which $\frac{\phi_0}{\Phi} = \frac{(R)}{(R) + K_M}$ we obtain by the insertion of $\frac{\Phi}{\phi} = \frac{v_0}{v} \cdot \frac{(R) + K_M}{(R)}$ in the above equation:

$$K_{M_1} = \frac{(F) \cdot K_M}{((R) + K_M) \cdot \left(\frac{v_0}{v} - 1\right)}$$

By means of this equation, Michaelis and Menten calculated, from the experimental data given above, the values 0.058 and 0.089, respectively, for the dissociation constants of the compounds of saccharase with frustose and glucose, the value 0.0167 determined for K_M being taken as a basis; however, because of the considerable sources of possible error in the method of calculation, these values must be regarded as merely indicative. The figures obtained by the authors in a similar manner by investigating the influence of other substances (sugars and glycerine) for the dissociation constants of their saccharase compounds or for their reciprocal values, which give the affinity to the enzyme, are brought together with those given above in Table XIV.

In a later investigation, Michaelis and Pechstein [1] succeeded in demonstrating that no affinity is to be recognized between the saccharase itself and glycerine, and possibly also the ordinary sugars which are not cleavage products of cane sugar. The authors make a distinction between two possible types of inhibition, according to whether the inhibiting substance is capable of reducing the concentration of the enzyme-substrate compound, as a result of its affinity to the enzyme itself, or whether it influences the decomposition velocity of this compound.

[1] Bio. Z. **60**, 79 (1914).

TABLE XIV

Affinity of Saccharase to Sugars

Example	Dissociation Constant K_M	Affinity Constant $\frac{1}{K_M}$
Cane sugar	0.0167	60.0
Fructose	0.058	17.0
Glucose	0.089	11.0
Mannose	About 0.083	12.0
Glycerine	0.075	12.0
Mannite	0.22	4.5
Lactose	0.5	Approaching 0.0

Differing Types of Inhibition.—In the equation

$$v = k \cdot \phi$$

which represents the proportionality assumed between the reaction velocity and the concentration of the enzyme-substrate compound, the two possible types of inhibition are expressed by a diminution either of the magnitude of ϕ or of that of k. We are capable of discriminating between these two possibilities in a given case, in so far as the inhibition coefficient $h = \frac{v - v_1}{v}$ in the first case, i.e., in a distribution of the enzyme between substrate and inhibition body, varies with the concentration of the substrate, whereas its value in the second case is found to be independent of the substrate concentration.[1]

The experimental procedure instituted by Michaelis and Pechstein consisted, accordingly, in investigating the inhibition coefficients of various addition substances with varying substrate concentrations. In the cases in which an affinity of the enzyme existed there occurred merely a parallel displacement of the activity-pS curve, but at the same time a change in the parameter of the curve, which likewise gives the dissociation constant (derived from one-half of the maximal velocity); for example, the action of the cleavage products of cane sugar, glucose and fructose, belongs

[1] Compare L. Michaelis and P. Rona, Bio. Z. **60**, 62 (1914).

to this type of inhibition.[1] In other cases, of a decrease in the decomposition velocity of the enzyme-substrate compound, the activity-pS curve undergoes an alteration in the sense that all its ordinates are reduced proportionally; in this way, for example glycerine and α-methyl glucoside exerted an inhibiting action and hence they were found to possess no affinity to saccharase itself.

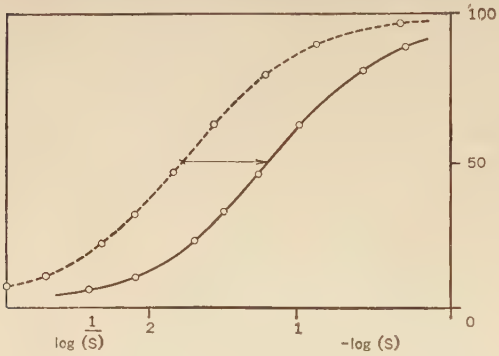


FIG. 5a.—Inhibition by affinity (fructose).

Figures 5a and 5b, given below, are taken from the experimental results recorded by Michaelis and Pechstein; they show the characteristic difference in the displacement of the activity-pS curve in both types of inhibition, 5a showing the influence of fructose, 5b that of the α-methyl glucoside upon cane-sugar cleavage by saccharase.

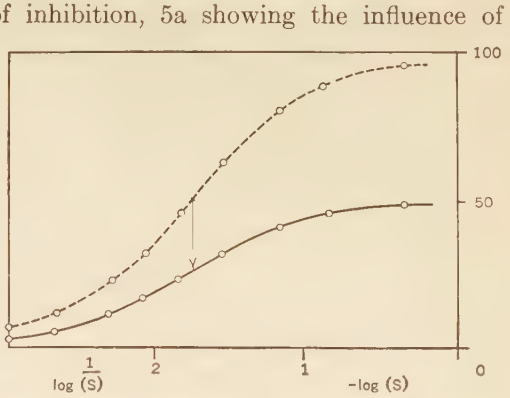


FIG. 5b.—Inhibition of decomposition velocity (α-methyl glucoside).

Reaction Equation of Saccharase Action.—The values obtained for the magnitude of the separate affinities of saccharase to cane sugar, to fructose and to glucose have enabled Michaelis and Menten [2] *to predict and describe the reaction process of enzymic cane-sugar hydrolysis on the basis of the law of mass action alone,* with

[1] According to further investigations by R. Kuhn and H. Münch, however, H. **163**, 1 (1927), the glucose contained in cane sugar possesses no affinity for saccharase, its influence acting only in the decomposition of the enzyme-substrate compound.

[2] Bio. Z. **49**, 333 (1913).

only the aid of the arbitrarily chosen constant, which corresponds to the given enzyme quantity. They proceed from the assumption that all the equilibria present are very rapidly established and can be dealt with on the basis of the law of mass action, and that the decomposition velocity of the saccharase-cane sugar will be found proportional to its concentration at any given time. If

Φ = the total enzyme concentration,
ϕ = the concentration of the enzyme-cane sugar compound,
ψ_1 = the concentration of the enzyme-fructose compound,
ψ_2 = the concentration of the enzyme-glucose compound,
(R) = the concentration of the free cane sugars,
(F) = the concentration of the free fructose,
(G) = the concentration of the free glucose,
a = the initial concentration of the cane sugars, and
x = the quantity of cane sugar transformed in time t,

then according to the law of mass action, we have:

$$(R)\cdot(\Phi - \phi - \psi_1 - \psi_2) = K_M\cdot\phi$$
$$(F)\cdot(\Phi - \phi - \psi_1 - \psi_2) = K_F\cdot\psi_1$$
$$(G)\cdot(\Phi - \phi - \psi_1 - \psi_2) = K_G\cdot\psi_2$$

By means of the definition given by the equation $v = \frac{dx}{dt}$ for the decomposition velocity at the given time t, and the ratios existent at this time, namely,

$$(R) = a - x \quad \text{and} \quad (F) = (G) = X$$

we obtain the following relation by combination of the above three equations and integration by a method which is not given in detail here:

$$K = \frac{1}{t}\cdot\left(\frac{1}{a} + \frac{1}{K_F} + \frac{1}{K_G}\right)\cdot a\cdot ln\frac{a}{a-x} + \frac{X}{t}\cdot\left(\frac{1}{K_M} - \frac{1}{K_F} - \frac{1}{K_G}\right)$$

According to the example of Henri,[1] they represent the reaction velocity as being the sum of a logarithmic and a linear function of the transformation. If, in the equation, the affinity of the enzyme to its substrate is placed equal to the affinity of the enzyme

[1] Lois générales de l'action des diastases, Thèse. Paris, Hermann, 1903.

to either of the cleavage products, i.e., $K_M = K_F = K_G$, then the resulting equation is that of a mono-molecular reaction. But, on the other hand, if K_M is assumed to be very large in proportion to K_F and K_G, then there is a direct proportionality between the transformation and the time.

By inserting the values found by Michaelis and Menten for their saccharase solution, viz., $K_M = 0.0167$, $K_F = 0.058$ and $K_G = 0.089$ we obtain from the above equation:

$$k = \frac{1}{t} \cdot (1 + 28a) \cdot 2.303 \cdot \log \frac{a}{a - x} + \frac{32 \cdot X}{t}$$

The experimental verification of the calculated ratios showed that they do in fact appear to reproduce with considerable accuracy the reaction process of cane-sugar cleavage. The constancy of the value for k proved to be adequate and independent of the cane-sugar concentration in the separate series of experiments. The same result was obtained in subsequent experiments by Willstätter, Graser and Kuhn [1] as well as by Euler. The Michaelis equation, accordingly, is to be regarded as the best expression thus far derived for the kinetics of cane-sugar hydrolysis. Objections raised by Arrhenius [2] cannot as yet be adjudged with certainty; moreover, the discrepancies pointed out by him in the experimental results which he obtained with very dilute cane-sugar solutions may be attributed to errors of determination.

The representation by Michaelis and Menten, according to which the velocity of cane-sugar hydrolysis is determined solely by the concentration of the saccharase-cane sugar compound, i.e., by the amount of enzyme bound on the cane sugar, and according to which the decomposition velocity of this compound is only of subordinate importance, holds good meanwhile only for the comparison of reaction velocities with the specified hydrogen number.

Action of Hydrogen-ions.—According to the investigations by Kuhn [3] the fraction of saccharase combined with the cane sugar is independent of the hydrogen number; [4] when, in spite of

[1] H. **123**, 1 and particularly p. 69 (1922).

[2] Z. Ang. **36**, 455 (1923).

[3] Naturw. **11**, 732 (1923).

[4] According to H. v. Euler and R. Josephson, however, this is only true for the alkaline branch of the activity-*p*H curve. H. **134**, 50 (1924); **155**, 1 (1926).

this, the velocity of hydrolysis, as thoroughly explained in Chapter III, is found to be in great measure dependent on the H-number, then this phenomenon is most probably due to the fact that *the H-ions influence the decomposition velocity of the enzyme substrate compound.* In consequence of the equality of the affinity constants of the enzyme with a varying *p*H, we shall find with different enzyme quantities, purely on the basis of Michaelis' premises, varying reaction constants with any other than the optimal H-number employed.

Temperature Relations.—A further circumstance by which the velocity of enzymic reactions is determined is based upon their *temperature dependence.* The influence of temperature is concerned almost exclusively with the velocity with which the equilibrium of the enzymic process is established, but not, on the other hand, the equilibrium point itself; in fact, the velocities increase in approximately the same degree as has been observed with non-enzymic processes, i.e., with a temperature increase of about 10°, the increase is generally two or three fold.

The most reliable temperature coefficients of the principal enzyme reactions, which show the relation of the reaction velocities at the beginning and at a temperature 10° higher, namely, $\frac{K_{t+10}}{K_t}$ are given in Table XV.

Some of the values presented here, however, have been obtained without sufficient regard for the influence of hydrogen number and consequently it can hardly be claimed that the table is correct throughout. It may be added that in many cases sufficient significance has not been attached to the increased destruction of the enzyme which occurs at higher temperatures, this destruction process competing with that of the increase in reaction velocity. This is true particularly for the region of higher temperatures, since here the increase of enzyme destruction with rise in temperature is much more pronounced than the increase of the transformation. For this reason the numerous reports in the literature concerning optimal temperatures for particular enzymic reactions are of problematic value; they vary ordinarily with the duration of the experiment and with the hydrogen number

Temperature Inactivation.—The process of enzyme inactivation with increasing temperature, which will be treated briefly here, has been the subject of much investigation; the assumption

that it follows a mono-molecular reaction course has been proved to be correct for a number of enzymes. For example, the heat inactivation of the emulsin of bitter almonds belongs in this category, according to Tammann,[1] and also, according to Madsen

TABLE XV

Temperature Coefficients of Enzymic Reactions

Enzyme	Substrate	Temperature Interval in Degrees C.	$K_t+10 : K_t$	Observer
Pancreas lipase	Ethyl butyrate	20–30	1.26	Kastle and Loevenhart [1]
Ricinus lipase	Olive oil	20–30	1.23	Nicloux [2]
Pepsin	Gelatine	25–30	1.95	Arrhenius [3]
Trypsin	Casein	20–30	5.30	Bayliss [4]
Erepsin	Leucyl glycine	25–35	1.67	Abderhalden [5]
Yeast saccharase	Cane sugar	25–35	1.61	Nelson and Bloomfield [6]
Maltase	Maltose	20–30	1.44	Lintner and Kröber [7]
Emulsin	Salicin	20–30	2.62	Tammann [8]
Pancreas amylase	Starch	30–40	2.0	Kendall and Sherman [9]
Malt amylase	Starch	20–30	1.96	Lüers and Wasmund [10]

[1] Am. **24**, 491 (1900).
[2] Soc. Biol. **56**, I, 701, 839, 868 (1904).
[3] Medd. fra Carlsberg Labor. 1881, p. 335.
[4] Arch. sciences biol. Petersbourg **11**, 261 (1904).
[5] H. **59**, 293 (1909).
[6] Am. Soc. **46**, 1025 (1924).
[7] B. **28**, 1050 (1895).
[8] H. **16**, 271 and particularly p. 321 (1891–92).
[9] Amer. Soc. **32**, 1087 (1910).
[10] Fermentf. **5**, 169 (1921–22).

and Walbum,[2] the heat destruction of proteolytic enzymes, of pepsin, lab and trypsin. Table XVI shows the results by Tammann for the destruction of almond emulsin at 60° C; the inactivation constants given in the last division of the table are calculated from the equation

$$k_c = \frac{1}{t} \cdot ln \frac{k_a}{k_t}$$

in which k_a signifies the initial reaction velocity and k_t that measured after the duration, t, of heating.

[1] Ph. Ch. **18**, 426 (1895); H. **16**, 271 and particularly p. 324 (1891–92).
[2] Consult in Sv. Arrhenius, Immunochemie, p. 57-59. Leipzig 1907.

TABLE XVI

HEAT INACTIVATION OF EMULSIN AT 60°

(*According to Tammann*)

Time (t) Hours	Relative Activity, Per Cent	$k_c = \frac{1}{t} \cdot ln \frac{k_a}{k_t}$
0.25	64.6	0.76
0.50	36.3	0.88
0.75	23.9	0.83
1.00	17.7	0.75

In other cases, on the contrary, as the inactivation of saccharase or the inactivation of blood catalase, the process appears to be less simple; it has been shown, particularly by the results of Willstätter, Graser and Kuhn,[1] that the temperature sensitivity is in great measure dependent on the degree of purity of the given enzyme, and hence consideration must always be given to the influence of concomitant substances in all conclusions concerning the course of the inactivation.

The special influences of temperature increase on enzymic cane-sugar hydrolysis have been exhaustively analyzed by Euler and Laurin.[2] On the basis of their findings, according to which the dissociation constant of the saccharase-cane sugar compound undergoes a relatively small increase with a rise in temperature—it increased only about 50 per cent between 1° and 39° C.—the conclusion follows that the increase of the reaction velocity in this case is to be attributed to an increased decomposition tendency on the part of the enzyme-substrate compound.

Enzyme and H-ion Catalyses.—It is of interest in this connection *to compare the phenomena of enzymic and H-ion catalysis of sugars and glucosides.* Euler has called attention to the fact that the given velocities of cane-sugar hydrolysis, as effected by H-ions and by saccharase, respectively, are attained in very different manner. In an example cited, in which the reaction velocities were equal, the affinity constant of the catalyzer-substrate

[1] H. **123**, 1 and particularly p. 72 (1922).
[2] H. **108**, 64 (1919).

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

compound amounted to 50 for the enzyme, and, on the other hand, to only 10^{-6} for the H-ion catalysis; accordingly, for the attainment of the given transformation in the second case, a relative catalyst concentration of ten million is required. Furthermore, the researches of Kuhn and Sobotka,[1] which have dealt with a comparison of H-ion and ferment catalysis, have led to the remarkable result that for a like concentration of the catalyst-substrate compounds, the ratio of the reaction velocities for the hydrolysis of saccharides and glucosides, whether by enzymes or by H-ions, is usually found constant; the assumption of a like concentration of the catalyzer-substrate compounds here, takes into account, necessarily, the varying affinity constants. The ratio of the reaction velocities is thus shown to be independent of the nature of the active catalyst, as is evident from Table XVII.

TABLE XVII

H-Ion and Ferment Catalysis

(*According to Kuhn and Sobotka*)

Substrate	Relation of Reaction Velocities for	
	H-Ions	Enzymes
Cane sugar : Raffinose	1.6	2 ± 1
α-Phenyl-: α-Methyl glucoside	25.0	28.0
α-Phenyl glucoside : Maltose	0.14	0.20

Now the researches of Euler and Laurin [2] have called attention to the fact that the temperature coefficients of the hydrolysis velocities for acid and for enzymic cane-sugar catalysis differ considerably, the temperature coefficient of the reaction through catalysis by H-ions being more than $2\frac{1}{2}$ times as great. The transformation increasing effect of a rise in temperature, which in the case of saccharase action concerns only the decomposition velocity of the enzyme-substrate complex, must therefore mani-

[1] Ph. Ch. **109**, 65 (1924).

[2] l. c.

fest itself likewise in the hydrolysis by H-ions, by a concentration increase of the intermediate reaction products, presumably because of an increase of the cane-sugar kations; such an increase would, in fact, result in this case in an increase of concentration of the catalyst-substrate compound, despite an unaltered concentration of the H-ions themselves.

Mechanism of Enzyme Actions.—The collected results obtained by researches, particularly in the recent decades, as to the course followed by enzymic reactions, leads to a somewhat more thorough examination and a test as to the validity of *the special views which have been expressed concerning the mechanism of these processes.* The best recognized theories are more or less in agreement as to the assumption that an intermediate compound of catalyst and substrates must be formed in enzymic reactions as in other catalyses; nevertheless, the explanations offered by individual researchers for the formation and the mode of combination of enzyme and substrate are widely divergent and some of these will be presented here.

As has been shown in the foregoing and particularly as supported by the investigations of the Michaelis school, the union of enzyme and substrate (at least for the specially investigated case of cane-sugar hydrolysis) is considered as an ionic reaction, i.e., as a reaction between chemical groups on the basis of their specific electrical charge. The application of the mass action law to the relations which arise between the enzyme and the substrate, as expressed in the fundamental work of Michaelis, has the further premise that the substances which take part in the reaction are present in a homogeneous solution and that the mass of their combination is determined exclusively by their concentration in the solution.

In opposition to these electro-chemically derived theories are the hypotheses by which Bayliss [1] has sought to explain the nature of enzymic processes, these hypotheses having been developed from a predominantly colloidal chemical point of view. The hypotheses advanced by Bayliss conceive the nature of *enzyme action* as a more or less *specific adsorption process*, which constitutes a preliminary stage for the *subsequent chemical reaction at the*

[1] The Nature of Enzyme Action, 5th Ed., London, 1925; Biochem. Journ. **1**, 175 and particularly p. 222 (1906); J. Physiol. **46**, 236 and particularly p. 251 (1913).

surface of the adsorbed particles. The primary combination of enzyme and substrate, according to this, is to be ascribed less to a definite chemical affinity between the enzyme and substrate; the reaction acceleration induced by the enzyme depends more upon an increase in concentration of the substances concerned at the surface of the enzyme particle; in hydrolytic processes, for example, substrate and water are adsorbed on the surface of the enzyme particle. According to this view, then, the actual transformation takes place at this interface, which is likewise subject to the validity of the mass action law, for the measure of which, nevertheless, the existing adsorbed portion of the substances concerned is to be regarded as decisive. When in certain cases, e.g., in the hydrolysis of stereo-isomeric glucosides, a definite optical specificity of enzyme action is observed, this phenomenon is forthwith explained by Bayliss as being due to a specific, spacial arrangement of the reacting groups of enzyme and substrate (one placed above another); however, the chemical affinity is not responsible for the preference of only the one isomer, but the possibility of an intimate contact of the enzyme and the adsorbed substrate, occasioned by the special steric arrangement.

The combination of enzyme and substrate has been regarded still less in a chemical sense in the researches of Fodor [1] and Abderhalden and Fodor,[2] which have already been described in Chapter I. Whereas, the theories developed by Bayliss seek to explain the selective action of the enzyme by the assumption of a specific adsorption of the substrate, these investigators proceed upon the theory of a general adsorption of the substrate by the enzyme; the combination of the enzyme and substrate is not regarded as specific, but rather the cleavage into the resulting products.

Application of the Mass Action Law.—These theories, however, are *not supported* by the experimental findings of the more recent enzyme studies; it may be said in general that *they are not in themselves alone capable of explaining adequately the nature of enzymic processes.* The investigations described in the foregoing paragraphs of the chapter have proved that in a series of cases, especially for the action of carbohydrate splitting enzymes, the course of enzymic reactions can be expressed with fair accuracy on the

[1] Das Fermentproblem. Dresden und Leipzig, 1922.
[2] Fermentf. **1**, 533 (1916); **2**, 74 (1917).

basis of the mass action law,[1] and further, that the total concentration of the substrate is responsible for the reaction velocities and not merely a definite adsorbed fraction of the substrate. The equilibrium between the enzyme and the substrate is determined in these cases as in a homogeneous solution by the dissociation constants of the enzyme-substrate compound. On the other hand, the results obtained in the researches of Willstätter [2] in the preparative purification of many enzymic substances, have demonstrated that, for the enzymes themselves, the active mass is determined solely by their concentration, and they are not at all, or not materially, influenced by alteration of the degree of dispersion and by surface variations.

The view that a definite surface development is to be regarded as an essential preliminary for the activity of enzymes appears, it is true, to be supported by certain properties, especially in the more complicated reaction systems, as in that of the fat cleaving enzymes. Thus pancreatic lipase is found to be only partially active in some of its adsorbates, as in alumina and kaolin adsorbates, and hardly active at all in others, as the adsorbates of tristearin and cholesterin. However, these phonomena are not to be attributed to an injury of the adsorption ability but rather to an injury or repression of its specifically reactive grouping, for, in fact, the enzyme can be elutriated in a state of undiminished activity from the adsorbates mentioned.

At all events, the kinetics of enzymic fat cleavage permits the recognition of a marked dependence upon the establishment of a definite adsorption state: the water-soluble enzyme, animal lipase, requires for contact with the water-insoluble fat, the interposition of certain concomitant or addition substances with pronounced adsorptive properties with respect to both the enzyme and the substrate; the mere production of a good emulsion, i.e., a mechanical suspension of the substrate in the enzyme solution, is not sufficient in this case. But it is not the surface development of the enzyme itself or of its substrate which is responsible for the existing activity, it is rather the adsorption affinities of the non-specific substances which do not share in the reaction itself, but merely facilitate the reaction. In the case of the

[1] See on the other hand the work by S. G. Hedin, H. **146**, 122 (1925); **154**, 252 (1926).

[2] Compare B. **55**, 3601 and particularly p. 3605 (1922).

water-insoluble plant lipases, on the other hand, the adsorptive union to a non-soluble reaction intermediary is already accomplished intracellularly and is so pronounced that the action of the enzyme remains closely connected with the surface condition of this bearer. Moreover, in these special cases, the activity of the enzyme depends not upon the degree of its own dispersion but upon the adsorption ability of the more or less closely associated intermediaries. These intermediaries become effective, with respect to the substrate, or in the case of animal lipase with respect to the enzyme and substrate. The application of the mass action law alone is not sufficient means for the comprehension and description of such non-homogeneous reaction systems, even if it must be assumed that the nature and the course of the essentially fermentative transformation in homogeneous and in non-homogeneous systems are fundamentally the same. For the recognition of this, meanwhile, the study of simple, homogeneous reaction systems is of value.

The Specific Reactive Group.—The actual existence of a specific substrate-binding group in the enzyme molecule is generally accepted today. With regard to its chemical nature, however, there is no evidence beyond that which has been obtained by indirect methods. Nevertheless, some of the results have been sufficiently indicative to warrant serious mention. A number of independent experimenters working with entirely different enzymes have obtained evidence which would suggest an aldehydic type of reactive group.

Euler and Svanberg,[1] for instance, working with saccharase, have found that the enzyme is strongly inhibited by mercury and silver salts and that there is a uniform relation between salt concentration and toxic action. The toxic action, further, is quantitatively reversible. The substrate exerts a pronounced protective action against inactivation. Aniline derivatives have a toxic effect and their relative toxicity seems to be comparable to their relative affinity to formaldehyde (formation of a Schiff base).

Woker [2] has reported a "peroxydase" and "catalase" reaction of formaldehyde and acetaldehyde and has postulated an enzyme model on the basis of an aldehyde grouping.

The same worker has suggested a similar model for amylase

[1] Fermentf. **3**, 330 (1920); **4**, 29, 90, 142 (1920–21).
[2] B. **47**, 1024 (1914).

action and has reported a degradation of starch by formaldehyde. The latter, however, was shown to be incorrect, at least so far as any profound degradation was concerned.[1] Olsson [2] has found that the action of malt amylase is markedly inhibited by aldehyde reagents such as phenylhydrazine, hydroxylamine, semicarbazide, sodium sulfide and potassium cyanide.

With regard to the action of erepsin (polypeptidase), Josephson and Euler [3] have shown that its action also is inhibited by the presence of aldehyde reagents. They consider that an aldehyde group of the erepsin molecule attaches itself to the free amino acid group on the end of peptide chain. Waldschmidt-Leitz and Rauchalles[4] have carried out experiments which would support a similar inference. It is shown that the *p*H curve of glycyl glycine hydrolysis by intestinal erepsin coincides almost exactly with the *p*H curve of the condensation velocity of glycyl glycine with glucose.[5] The conclusion seems logical that the peptide in both cases reacts with a similar chemical grouping.

At best, however, the aldehyde nature of the reactive group is hardly more than speculative, being based on analogy and environmental conditions of its reactions. A conclusive decision must await more positive evidence.

Euler's Conception.—Euler and Josephson [6] have also expressed a further hypothesis for the special case of cane-sugar hydrolysis by saccharase, which attempts to explain the chemical process of the formation and cleavage of saccharides. Saccharase is looked upon because of its amphoteric nature and its behavior to protolytic enzymes, as well as because of the results of preparative researches, as an amphoteric protein derivative, provided with a special cane-sugar binding group. The basic principle which distinguishes the Euler theory may be expressed

[1] See Walton, "A Comprehensive Survey of Starch Chemistry." 1928, part 2, p. 96-98.

[2] H. **117**, 97 (1921).

[3] H. **162**, 85 (1927).

[4] B. **61**, 645 (1928); see also K. Josephson, B. **61**, 1276 (1928).

[5] The condensation of aldehyde sugars with amino acids is a well-known reaction. This reaction leads to an equilibrium, which corresponds to a stronger increase of condensation with increasing alkalinity. The condensation of glucose with peptides is characterized by a wholly different dependence on the H-ion concentration, that is, it exhibits a definite optimum at *p*H 8.

[6] H. **133**, 279 (1923–24).

by saying that *the cleavage of the cane sugar does not occur at the same point of the enzyme molecule at which the binding of the substrate takes place.* A definite discharge through a dissociated group of the protein part of the saccharase is considered as responsible for the cleavage in analogy to the action of hydrogen-ions. The marked difference of affinity between H-ions and enzymes is ascribed to the fact that because of the union of the cane sugar with the enzyme in this case, the probability of a discharge of the substrate must be much greater. This theoretically important viewpoint, however, has not been definitely established.

Enzymic Equilibria and Syntheses.—The kinetics of enzymic reactions has thus far been considered solely on the basis that they are operative only in the direction of hydrolysis and that the opposing, synthetic reactions are not to be considered. However, the mass action law shows that not only the velocity but also the direction of reversible processes is determined by the concentration of the substances involved; this holds true not only for the velocity of the first reaction, but also for that of the opposite, contrary reaction. According to this the position of equilibrium of a reversible reaction is determined by the concentration of all the substances involved, and accordingly by the relation of the velocities with which the reaction proceeds in both directions. According to the dynamic concept the transformation at the point of equilibrium proceeds in both directions with the same velocity; hence, the equilibrium constant is given by the relation of the two constants of the reaction velocity, e.g., of the hydrolytic and the synthetic:

$$K = \frac{k_1}{k_2}$$

If the velocity of ester splitting is expressed by the relation

$$v_1 = k_1 \cdot (\text{Ester}) \cdot (\text{Water})$$

and that of ester formation by the analogous expression

$$v_2 = k_2 \cdot (\text{Alcohol}) \cdot (\text{Acid})$$

then we derive therefrom for the equilibrium point, in which $v_1 = v_2$, the equilibrium constant

$$K = \frac{k_1}{k_2} = \frac{(\text{Acid}) \cdot (\text{Alcohol})}{(\text{Ester}) \cdot (\text{Water})}$$

The equation shows that, for example, an increase in concentration of the cleavage products, of acid or alcohol in the present case, results in a retardation of ester saponification, and, further, in a retardation which must be distinguished from the type already described, i.e., the type which depends upon the affinity exerted by the catalyst. The equilibrium point of such a reversible reaction is independent of the velocity with which it has been attained, as shown by the given equation and also as verified experimentally. The equilibrium point, likewise, is not influenced by the presence of an ideal catalyst, which unites with only a negligible portion of the reacting components and moreover is not influenced by the concentration of the catalyst.

Van't Hoff [1] first pointed out in 1898 the theoretical possibility that enzymes may catalyze reactions not only in the direction of hydrolysis, but also in the direction of synthesis. His conclusions were experimentally confirmed very shortly thereafter by Croft Hill,[2] who was successful in effecting the first enzymic synthesis of a disacccharide.

The recognition of the synthesising action of enzymes lent interest also to the question as to whether they were able to effect displacements in the ordinary equilibria states of chemical reaction systems, i.e., whether enzyme equilibrium is to be regarded as identical with that effected by other catalysts. According to the laws of thermodynamics, it was to be expected that an enzymic catalyst, incapable of binding an essential portion of the reacting components, would cause no alteration of the usual equilibrium point. The results obtained with enzymic equilibria have shown meanwhile that this requirement, apparently, is not fulfilled in all enzymic reactions, even though in many cases the point of the non-enzymic equilibrium cannot be definitely determined.

The first extensive researches upon the position of enzymic equilibria date from 1903 to 1906 and deal with the cleavage and synthesis of esters. Thus Pottevin [3] first described the enzymic synthesis of an oleic acid ester, methyl oleate, from oleic acid and methyl alcohol, by means of pancreatic lipase; this study was carried out with especial relation to the influence of various

[1] Z. a. Ch. **18**, 1 (1898).
[2] Soc. **73**, 634 (1898).
[3] C. r. **138**, 378 (1904); Ann. Inst. Pasteur **20**, 901 (1906).

enzyme quantities upon the degree of synthesis attained. It was found, as shown in Table XVIII, that there is no special dependence of the equilibrium point upon the quantity of catalyst employed.

TABLE XVIII

SYNTHESIS OF METHYL OLEATE

(*According to Pottevin*)

Equimolecular Quantities of Oleic Acid and Methyl Alcohol, 33°

Relative Enzyme Quantity	Per Cent Synthesis According to Days		
	1	3	20
1	8	56	84
2	12	66	82
5	21	66	84
10	42	74	85

Pottevin also investigated the synthesis of triolein, the trioleic glyceride, by pancreas lipase, with respect to dependence upon glycerine concentration. Later, Jalander [1] demonstrated for the formation of this fat that the equilibrium point obtained with a plant lipase from ricinus seed, was, within the experimental error, in agreement with the hydrolytic equilibrium. Furthermore, Taylor [2] has compared the hydrolytic equilibrium of triacetin by ricinus lipase with that obtained by H_2SO_4 at various substrate concentrations; only for the higher concentrations of the glyceride was there noted a displacement in the direction of lessened hydrolysis for the enzymic process. However, the comparability of the experiments described has not been fully ascertained. Table XIX shows the deviations found with larger quantities of ester.

The most extensive investigation of equilibria in enzymic ester hydrolysis has been carried out by Bodenstein and Dietz.[3]

[1] Bio. Z. **36**, 435 (1911).
[2] J. Biol. Chem. **2**, 87 (1906).
[3] Z. El. Ch. **12**, 605 (1906).

These experimenters studied the hydrolysis and synthesis of amyl butyrate under the influence of pancreatic lipase. According to their researches there occurs at a definite concentration of the substances involved an equilibrium which can be reached from either side of the equilibrium point and the position of which is

TABLE XIX

EQUILIBRIA IN THE ACID AND ENZYME HYDROLYSIS OF TRIACETIN

(*According to Taylor*)

Substrate Concentration Per Cent	Equilibrium in Per Cent Hydrolysis with	
	$0.5n$ H_2SO_4	Ricinus Lipase
0.5	88	86
1.0	82	79
2.0	78	70

independent of the enzyme quantity. With increasing substrate concentration, however, there is an alteration, contrary to the tenets of the mass action law, in the relation of the hydrolytic and synthetic reaction velocities, which is expressed by the equation

$$K \text{ (equilibrium constant)} = \frac{k_2 \text{ (hydrolysis)}}{k_1 \text{ (synthesis)}}$$

that is, the equilibrium constant is augmented. The change in the equilibrium constants with respect to their dependence upon the substrate concentration is shown by the following statement, according to Bodenstein and Dietz:

Substrate Concentration Normality	Equilibrium Constant K
0.05	0.45
0.10	0.74
0.20	1.12

Comparison with acid catalysis showed that in the case of the enzymic reaction the equilibrium was considerably displaced in

favor of the hydrolysis; e.g., in the example studied there was an ester formation of 75 per cent for the enzyme in contrast to 85.5 per cent for the action of H-ions, and the equilibrium constant of the acid catalysis was found to be 1.12. The equilibrium displacement observed under action of the enzyme is interpreted by Haber [1] by assuming that there is an action of special surface forces in the existing macro-heterogeneous system, and by Freundlich [2] as a concentration displacement on the interface of the enzyme particles.

Armstrong [3] has expressed the view that enzymes are capable of synthesizing only those substances which they do not hydrolyse, and that accordingly a distinction must be made between hydrolysing and synthesizing enzymes. This is based on the results obtained by Emmerling [4] according to which the disaccharide obtained by Croft Hill through the action of yeast extract on grape sugar is to be identified with isomaltose, hence with a substance which is not hydrolysed by the maltase of yeast, similar observations having also been made by Fisher and Armstrong.[5] But this theory has not been supported by the extensive researches of Bourquelot [6] and his school, not even with the carbohydrate splitting enzymes, which they are held primarily to concern; thus it has been shown that, contrary to the particular theories of Armstrong, the glucosidase of bitter almonds is capable of forming the same glucosides which it hydrolyses. Furthermore, in the action of glucoside-splitting enzymes, according to Bourquelot, there is a formation of true equilibria, which can be reached from both sides, as well by the hydrolytic as by the synthetic procedure. Thus in an example of the hydrolysis or synthesis of β-methyl glucoside by emulsin in 30 per cent methyl alcohol, the velocity of the two processes nearly coincided, hence there was obtained for the equilibrium constant $K = \frac{k_2}{k_1}$ the value 1, corresponding to a hydrolysis or synthesis of 50 per cent.

[1] See C. Oppenheimer, Die Fermente und ihre Wirkungen, 4. Aufl., Leipzig. 1913, **2,** 937.

[2] Kapillarchemie, 2. Aufl., Leipzig, 1922, p. 1054.

[3] Proc. Roy. Soc. B. **76,** 592 (1905).

[4] B. **34,** 600, 3810 (1901).

[5] B. **35,** 3144 (1902).

[6] See A. ch. (9) **7,** 153 (1917).

The results of a series of experiments are assembled in Table XX.

TABLE XX

HYDROLYSIS OR SYNTHESIS OF β-METHYL GLUCOSIDE

(*According to Bourquelot*)

Time, Days	Synthesis		Hydrolysis	
	Observed Rotation, Minutes	Decrease	Observed Rotation Minutes	Increase
0	+64	0	−42	0
1	+58	6	−36	6
2	+48	16	−26	16
4	+40	24	−18	24
8	+28	36	− 6	36
13	+16	48	+ 6	48
18	+12	52	+10	52
.....	Final Value	.....	Final Value	

This equilibrium constant, however, holds only for the β-glucoside, the velocity of transformation of α-glucoside being entirely different; thus, for example, a constant K_α of 0.484 was found for the action of maltase on α-ethyl glucoside with an alcohol content of 20 per cent and K_β of 0.308 for the action of emulsin on β-ethyl glucoside.

This difference was confirmed likewise by the simultaneous action of both enzymes on the mixture of substrates; the result coincided with that which had been calculated from the given constants on the basis of the mass action law.

Equilibrium Point of Enzyme Reactions.—The above result obtained by Bourquelot,[1] according to which both the synthesis and the hydrolysis of methyl glucoside by emulsin under the given conditions proceed with the same velocity, holds, however, only for the enzymic process, the equilibrium in acid catalysis being different from that occurring in enzymic catalysis. Thus Euler and Josephson [2] have based a calculation on the experimental

[1] See particularly J. Pharm. Chim. (7) **10**, 361, 393 (1914).

[2] Arkiv. f. Kemi. **9**, Nr. 7 (1924).

results of Bourquelot and have obtained therefrom the value 0.25 for the equilibrium constant.

$$K_{(H^{\cdot})} = \frac{(\text{Alcohol}) \cdot (\text{Glucose})}{(\text{Water}) \cdot (\text{Glucoside})}$$

According to Euler [2] the *equilibrium position of enzymic reactions* is not determined, as in ideal catalysis, by the concentration of the substances concerned, but by *the relation of their affinities to the enzyme itself.* If, for example, the affinity of the enzyme to the glucoside is expressed by

$$K_1 = \frac{(\text{Glucoside} - \text{Enzyme})}{(\text{Glucoside}) \cdot (\text{Enzyme})}$$

and its affinity to the cleavage product by

$$K_2 = \frac{(\text{Glucose} - \text{Enzyme})}{(\text{Glucose}) \cdot (\text{Enzyme})}$$

the equilibrium position may then be calculated by inserting the affinity constants in the conversion equation. Thus, the equilibrium position at which the velocities of the hydrolytic and synthetic reactions coincide is represented by the equation

$$k_1 \cdot (\text{Glucoside} - \text{Enzyme}) \cdot (\text{Water}) = k_2 \cdot (\text{Glucose} - \text{Enzyme}) \cdot (\text{Alcohol})$$

wherein k_1 and k_2 represent the velocity constants of the two concurrent reactions; upon inserting the affinity constants of the enzyme we have

$$k_1 \cdot K_1 \, (\text{Glucoside}) \cdot (\text{Water}) = k_2 \cdot K_2 \cdot (\text{Glucose}) \cdot (\text{Alcohol})$$

The equilibrium of the enzymic reaction which is given by

$$\frac{k_1}{k_2} = \frac{K_2}{K_1} \cdot \frac{(\text{Glucose}) \cdot (\text{Alcohol})}{(\text{Glucoside}) \cdot (\text{Water})}$$

coincides accordingly with the equilibrium of the ideal catalysis, which corresponds to

$$\frac{(\text{Glucose}) \cdot (\text{Alcohol})}{(\text{Glucoside}) \cdot (\text{Water})}$$

only when the affinity of the enzyme to its substrate equals the sum of its affinities to the cleavage products, or when $K_1 = K_2$. Only in this case, as previously elucidated, does the course of enzymic reaction follow the equation of the first order.

[1] H. **52**, 146 (1907).

Taking into consideration the hypotheses just developed, Euler and Josephson have considered for the special case of the equilibrium position of β-methyl glucoside, as determined by Bourquelot, that the affinity of the emulsin to the glucose must be about four times as great as that to β-methyl glucoside, since it was found that $\frac{K\text{ (Synthetic)}}{K\text{ (Hydrolytic)}} = \frac{1}{0.25}$; as a matter of fact, calculations based on other investigations have given the value 3.3 for the relation of the affinity constants of the enzyme to glucose and to β-methyl glucoside.

The notable researches of Rosenthaler [1] as well as of Nordefeldt [2] upon the asymmetric synthesis of mandelic acid nitrile by emulsin will be described in Chapter VI. According to the observations of Bayliss [3] and of Nordefeldt, the equilibrium position for the enzymic synthesis of mandelic acid nitrile from benzaldehyde and hydrocyanic acid and that for the non-enzymic synthesis, i.e., that catalyzed by H-ions, is practically the same.

Meanwhile the accounts given in the literature as to enzymic syntheses do not confine themselves to the building up of esters or of simple carbohydrates and glucosides. There are occasional reports made also of the reverse formation of higher carbohydrates from simple sugars,[4] and of the production of definite equilibria, e.g., in the hydrolysis of starch,[5] under the action of enzymes, but the interpretation of these findings as synthetic processes is inadmissible in view of the complicated reaction systems under consideration. The same objection may be raised with respect to numerous observations as to the synthesis of protein or protein degradation products.[6] A synthetic combination of protein structural units by means of enzymes has not been positively demonstrated so far; the unsatisfactory experimental bases which are advanced as proof of such syntheses may be interpreted to have other meanings.

[1] Bio. Z. **14**, 238 (1908); **17**, 257 (1909).

[2] Bio. Z. **118**, 15 (1921); **131**, 390 (1922).

[3] J. Physiol. **46**, 236 (1913).

[4] Compare M. Cremer, B. **32**, 2062 (1899).

[5] Compare J. Wolff and A. Fernbach, C. r. **137**, 718 (1903); L. Maquenne, Bl. **35**, 1 (1906); E. C. Kendall and H. C. Sherman, Am. Soc. **32**, 1087 (1910).

[6] Compare V. Henriques and J. K. Gjaldbaek, H. **71**, 485 (1911); **81**, 439, (1912); **83**, 83 (1913); E. Abderhalden, Fermentf. **1**, 47 (1914).

V. ENZYMIC REACTION SYSTEMS

(Activation and inhibition)

Zymogens.—The opinion frequently advanced that some enzymes, in particular those of the animal organism, are not given off from the cells in the active condition, but in the form of inactive pre-stages, as "pro-enzymes" or "zymogens," is by no means of recent advent. Some of the earliest observations of this nature are found in the researches of Hammarsten,[1] of Langley,[2] and of Ebstein and Grützner,[3] according to whose views pepsin as lab is first secreted from the mucous membrane of the stomach in an inactive form; the HCl of the stomach is then supposed to transform this into the active form. It has been demonstrated, however, that this activation phenomenon is non-specific, and that it depends merely upon the establishment of a favorable acidity for activity of the pepsin or lab. In the same way many other conceptions of the older literature with respect to the activation of enzymic reactions have been traced or can be traced to a disregard of the influence of the H-number.

Aside from this observed influence of hydrogen- or hydroxyl-ions upon the activity of enzymes, which has already been discussed, *the existence of a certain number of typical activations can be demonstrated;* these are effected by the addition of special activators. But all hypotheses, according to which enzymes are formed in the cells, in the form of zymogens, have, as yet, proven themselves to be inadmissible. The phenomenon of enzyme activation by means of added substances (both specific and non-specific activation) will be described here with a few characteristic examples.

Activation by Inorganic Ions.—A typical example of simple *activation by inorganic ions* is given by the animal, starch-splitting

[1] R. Maly's Jahresbericht über die Fortschritte der Tierchemie **2**, 118 (1872).

[2] J. Physiol. **3**, 269 (1882).

[3] Pflüg. Arch. **8**, 122, 613 (1874).

enzymes. Earlier views as to the existence of an amylase zymogen, for which the lack of quantitative methods of determination may be partly responsible, have been rechecked and corrected, particularly by the researches of Bierry, Giaja and Henri.[1] From these researches it is evident that animal amylase, e.g., that of the pancreatic secretion, requires the presence of definite inorganic ions, particularly chlorine ions, for its action, and that the salt-free, dialysed enzyme solutions are inactive. This phenomenon, which has since been carefully investigated a number of times, distinguishes animal from vegetable amylase, according to Bierry;[2] with the latter, e.g., with the enzyme of germinating barley, no appreciable activation by salt ions was detected. On the other hand, Haehn and Schweigart[3] have shown that in the case of the amylase of potatoes this enzyme is identical with animal amylase in its relation to neutral salts.

According to Michaelis and Pechstein,[4] the active complexes of amylase and anions are characterized by widely varying dissociation constants; the stability of the compound of enzyme and ion appears, for example, to decrease in the following sequence of ions: $NO_3' Cl', Br' SO_4''$. This sequence is derived from the varying intensities of inhibition exerted by the ions on the enzyme, maximally activated by Cl-ions, under the assumption that a distribution of the enzymes takes place between the various ions and that the " chloride-amylase " represents the most active, but not the most strongly united ion compound of the enzyme. The fact that various acidity optima correspond to the individual amylase complexes is explained by the assumption that they represent different types of amylases. The differences observed, however, are considered by Hahn and Harpuder[5] as well as by Willstätter, Waldschmidt-Leitz, and Hesse[6] as being due to the combined salt-buffer activation; the influence of the activators is of varying magnitude with different hydrogen numbers.

The activation of amylase by inorganic ions, e.g., by sodium chloride, cannot be definitely compared with the influence exerted

[1] C. r. **143**, 300 (1906); Soc. Biol. **60**, 479 (1906); **62**, 432 (1907).
[2] Bio, Z. **40**, 357 (1912).
[3] Bio. Z. **143**, 516 (1923).
[4] Bio. Z. **59**, 77 (1914).
[5] Z. Biol. **71**, 287 (1920).
[6] H. **126**, 143 and particularly p. 150 (1922–23).

in general upon enzymes by hydrogen or hydroxyl-ions. Although, for example, it may be proved for saccharase, as explained in Chapter IV, that the affinity of the enzyme to its substrate does not appear to be influenced, within certain limits, by the H-number, and although for the action of other enzymes, e.g., the proteolytic enzymes, their dependence upon the reaction of the medium is to be interpreted as a different ionization of the substrate, nevertheless it cannot yet be determined whether the activating ions have an influence upon the affinity relations of the amylase itself, or whether they are not likewise capable of influencing the decomposition tendency of the substrate, as might be deduced from the observations (not yet fully confirmed) of Biedermann [1] concerning a salt hydrolysis of starch. Exact affinity comparisons will enable us to come to a decision regarding this question.

Specific Activation.—From the type of amylase activation which appears to be effected by various kinds of ions, and therefore to be non-specific, though it affects the entire operation of the enzyme and is therefore to be regarded as absolute, we must differentiate a *second type of enzyme activation*, which is characterized by the fact that it is capable of expressing itself only with respect to definite, particular affinities of an enzyme, or else with respect to *a definite stage of the reaction which it accelerates*; it is to be understood merely as relative and it is found to be much more specific, i.e., it is conditioned by the presence of *specific activators*. One of the best-studied examples of this is the activation of trypsin, the most important proteolytic enzyme of the pancreatic gland, by the enterokinase of the intestinal mucous membrane; this was discovered by Schepowalnikow,[2] working in the Pawlow laboratory. It was demonstrated by this investigation that pure pancreatic fistula secretion was entirely inactive with respect to the hydrolysis, for example, of true proteins, and that it was not until it reached the intestine, by the addition of one of the mucous membrane juices, namely, enterokinase, it became active; this phenomenon was looked upon as an enzymic process, i.e., as a transformation of the trypsin from its zymogen into the active form. However, it has become possible only at a very recent date to recognize with certainty the true nature of this activation

[1] Bio. Z. **129**, 282; **137**, 35 (1923).

[2] Inaugur. Diss. St. Petersburg, 1899; Referat in R. Malys Jahresbericht über die Fortschritte der Tierchemie **29**, 378 (1899).

process. That is, it has recently been shown in an investigation by Waldschmidt-Leitz [1] that the reaction between trypsin and enterokinase is based upon stoichiometric principles; and enzymic transformation of the "trypsinogen," as it is termed, does not occur. There is formed rather a compound of the enzyme and the activator, in considerable measure dissociated, whose formation requires a certain length of time. It was found possible, after successful activation, to separate the enzyme and activator by adsorptive methods.

Enterokinase, whose properties appear to resemble those of enzymic substances [2] is to be regarded as a specific activator of trypsin; the reports in the literature with respect to activations of this enzyme by other substances, such as lime or gall salts, have proved to be incorrect. But the influence of enterokinase concerns only certain cases of enzyme action, i.e., the activator posesses merely the significance of a substance which assists in the cleavage of certain special structural combinations, such as are present for example in genuine proteins. As Waldschmidt-Leitz and Harteneck [3] have shown, trypsin is capable, even without activation by enterokinase, of hydrolyzing a series of substrates, such as protamine or peptone, with considerable rapidity, even though its action in these cases is strengthened by the addition of an activator because of the dissimilarity of the structural groupings present in the substrates. This activation of trypsin by enterokinase, which is relative and specific, must be distinguished, therefore, from those described for animal amylase, even if future researches should prove that in the enzymic hydrolysis of starch also the influence of the activators is operative only with respect to certain stages of the reaction.

The phenomenon of trypsin activation finds its parallel in the activation of a plant protease, specifically in the activation of papain in the milky sap of the melon tree by hydrocyanic acid. In this case Willstätter and Grassman[4] have shown that the enzyme-hydrocyanic acid compound, whose formation likewise requires a certain length of time, is specific for the hydrolysis of certain simply constructed substrates, e.g., for peptone, while the papain does

[1] H. **132**, 181 (1923–24).
[2] E. Waldschmidt-Leitz, H. **142**, 217 (1924–25).
[3] H. **149**, 203 (1925).
[4] H. **138**, 184 (1924).

not require the intermediation of an activator for hydrolysis of other higher molecular proteins. It is not probable, however, that the action of papain-hydrocyanic acid and trypsin on the one hand, and of trypsin-enterokinase and papain, on the other, should be referable to the same specific structures.

Similar relations seem to exist in the activation of another important enzymic process, alcoholic fermentation. Harden and Young [1] have shown that the sugar fermenting action of zymase in yeast is due to the presence of a specific heat-resistant activator, "co-zymase," which may be removed, for example by extracting the dried fungus material with water; the remaining cell mass no longer possesses any fermentative power. From recent investigations by Euler and Myrbäck [2] the conclusion has been drawn that the conjunctive action of the " co-zymase " appears to be necessary for a wholly definite reaction stage of alcoholic fermentation, namely, for the intermediate formation of sugar-phosphoric acid ester, if not for a still earlier stage of the reaction.

Non-Specific Activations and Inhibitions.—A third and entirely different sort of enzymic activation is observed with animal lipases; it is comprehensively non-specific and only relative. The older views upon the subject, according to which lipase, for example, of the pancreatic gland, is secreted in the form of zymogen and is next transformed in the intestine into an active enzyme by the action of the gall, have been corrected by the researches of Willstätter, Waldschmidt-Leitz, and Memmen.[3] It has been demonstrated that the activation phenomena which are regarded as characteristic for the solutions of pancreatic lipase are entirely unspecific and are based merely upon a specially favorable adsorption condition for the contact of the water soluble enzyme with its insoluble substrate. " The action of the activators does not consist in the transformation of a lipase-zymogen into true lipase, for without activation the pancreas glands and their extracts effect fat cleavage in an acid medium with a velocity of the same order of magnitude as that which they attain in cleavage effected by activation in alkaline conditions." As activators for the cleavage of true fats by pancreatic lipase in an alkaline reaction, we have proteins, such as egg-

[1] Proc. Roy. Soc. B. **77**, 405; **78**, 368 (1906).
[2] H. **139**, 15 (1924).
[3] H. **125**, 93 (1922-23).

albumen, gall salts, or even lime soaps, the latter being formed upon addition of soluble calcium salts from the fatty acids developed. Their activating effect is due to the production of colloid particles which exert an adsorbent action with respect to the enzyme as well as to the substrate and thereby facilitate the reaction. The adsorbates which are formed, as the adsorbate

Albumen<Fat / Lipase , whose formation can also be demonstrated

experimentally with the individual adsorbates, e.g., by albumen and fat or by albumen and lipase, are designated by Willstätter as "complex adsorbates." Distinct from these are the so-called "coupled adsorbents," which proceed from the simultaneous action of several single activators, as the adsorption combination of calcium oleate—albumin; they are characterized by a specially

fat | lipase

favorable activating action; the combination of small amounts of such separate activators as lime soap or albumin results in the production of a much greater activation than with even large quantities of the components alone. Table XXI, which is taken from the investigations of Willstätter, Waldschmidt-Leitz and Memmen, illustrates the activating action of singly added substances, as well as the action of their combinations, upon pancreatic extract in an alkaline medium.

The magnitude and the sign of the activation are, however, largely dependent on the H-number and on the nature of the substrate employed. Proteins, lime salts or gall salts are good activators in an alkaline reaction yet are inhibitors in the acid region. On the other hand, the activating action of gall salts holds only for the hydrolysis of certain substrates, particularly for the natural fats, while they are found to be without influence or even to inhibit the cleavage of other substrates, such as the lower esters. Obviously in these cases the adsorbent action of the added substances is not the same for the enzyme and the substrate, but is effective with respect to only one of them and thus expresses itself in the direction of a reaction disturbance. As already set forth in Chapter III, the different effect of the activators may also decisively influence the dependence of enzyme action upon the H-number, e.g., with the natural enzyme solutions; thus the sup-

posed differences in the *p*H dependence of the stomach and pancreas lipases may be traced to the fact that only the first is found in its natural secretion accompanied by a concomitant substance, which represses its action in an alkaline medium and stimulates it in an acid medium.

TABLE XXI

Activation of Pancreas Lipase at *p*H = 8.9

$CaCl_2$, Mg.	Sodium, Glycocholate, Mg.	Albumin, Mg.	Oil Cleavage, Per Cent
.....	.....	.....	4.0
10	.....	.....	19.4
.....	10	.....	10.9
.....	80	.....	19.5
.....	.....	90	17.4
10	10	.....	13.6
10	.....	15	23.6
20	.....	30	27.2
.....	10	15	20.8
.....	20	30	22.8
10	10	15	23.4

It would exceed the limits of this book to go more fully into the numerous phenomena of activation and inhibition which have been observed with the fat-splitting enzymes. A common characteristic of all these is that they can be traced to adsorption actions, which are capable of either accelerating or hindering the enzymic reaction; whereas, the first mentioned activation phenomena—those of amylase or of trypsin—are to be explained as the action of a specific chemical influencing the affinity of enzyme and substrate. On the other hand one must discriminate between the non-specific inhibition phenomena that affect lipase action and the more specific inhibition influences that appear to concern the chemically active groups of enzymes. These inhibitions, to be discussed in the following pages, are reversible and are thereby distinguished from the irreversible destruction of the enzymes which takes place with hydrogen- or hydroxyl-ions or with a rise in temperature.

Toxic Influence of Heavy Metal Salts.—The poisoning action of heavy metal salts upon yeast saccharase has been extensively investigated by Euler and his co-workers. Thus Euler and Svanberg[1] first measured the inactivation of a saccharase preparation by sublimate, obtaining thereby, with increasing amounts of mercury, a typical asymptotic dissociation curve for the inactivation of the enzyme; this curve is given in Fig. 6.

According to Euler the form of the curve indicates that there is an equilibrium formed in the solution between the enzyme and the metal salt. It was successfully demonstrated also that the poisoning of the enzyme was accompanied by a corresponding decrease in the analytically determined heavy metal ions. Also it was shown that the inactivation was completely reversible; by introducing hydrogen sulfide into the poisoned enzyme solution, 96 per cent of the original enzymic activity was restored. Rather remarkably, cane sugar is able to exert a very considerable protective effect with respect to the poisoning of the enzyme by Hg, while its presence is without influence upon the inactivation of saccharase by Ag-ions, the latter inactivation being much stronger. Myrbäck[2] explains this difference in the protective influence of the substrate by the assumption that in the action of silver ions there does not occur, as is the case in Hg poisoning, a distribution of the ions between enzyme and substrate, but that the Ag reacts with the enzyme-substrate compound.

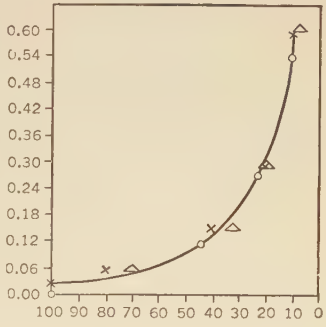


FIG. 6.—Inactivation of saccharase by sublimate.

Abscissae refer to relative inversion velocity; ordinates to mg. $HgCl_2$.

Equivalent Weights of Enzymes.—This inactivation of saccharase by Ag salts is further applied by Euler and Myrbäck[3] to a determination of the *equivalent weight* of the enzyme. Assuming that the pure saccharase has an inversion capacity (I_f) of 230—an assumption which, however, has been modified by the preparative results obtained by Willstätter and

[1] Fermentf. **3**, 330 (1920).

[2] Arkiv. f. Kemi **8**, 1, Nr. 29 (1923); C. 1924, I, 207.

[3] Z. exp. Med. **33**, 483 (1923).

Schneider [1]—the Ag equivalent of the enzyme, i.e., those quantities of saccharase which are required by 1 molecule of Ag for inactivation, is calculated to be 4,000 to 5,000. Myrbäck, on the other hand, has arrived at the lower value of 2,600 on the basis of the calculated dissociation constant of the Ag-saccharase-cane sugar compound.

However, our present knowledge does not allow us to judge of this magnitude with certainty. The values for the concentration of the pure enzyme, as used in the calculations by Euler, have not been verified, and it has also been shown that the Ag sensitivity of the saccharase appreciably increases with an increasing degree of purity, hence the given values for the Ag equivalent of the enzyme may prove to be too low.

Inactivation by Amines.—Observations upon the *inactivations of saccharase by organic bases*, particularly by aniline and *p*-toluidine, are further utilized by Euler and Myrbäck,[2] as a distinguishing feature of the nature of the specific, active grouping in the enzyme. This has been mentioned previously. The finding according to which this inactivation may be again annulled by the addition of aldehydes has led to the formulation of the theory that aniline and saccharase react to form an aldehyde-amine compound, i.e., a sort of Schiff's base. The analytically determined dissociation constant of the saccharase-aniline compound, which is not influenced by the presence of cane sugar, amounted to $2.5 \cdot 10^{-4}$ and the average of the determinations for the formaldehyde-aniline compound amounted to $18 \pm 3 \cdot 10^{-2}$, the aniline compound of the enzyme, accordingly, being less dissociated.

Other Toxic Agents.—It would take us too far afield to discuss here all the special reports in the literature concerning the inhibition of enzymic reactions by definite inorganic salts or by organic addition substances, especially since many of them no longer appear to be sufficiently genuine.

To be emphasized is the pronounced inhibiting action of sodium fluoride on animal lipase, which was first observed by Kastle and Loevenhart,[3] as well as the strong inhibiting action on catalase,[4] or

[1] H. **133**, 193 (1923–24).

[2] H. **125**, (1922–23); K. Myrbäck, Arkiv. f. Kemi **8**, 1, Nr. 32 (1924); C. **1924**, I, 208.

[3] Am. **24**, 491 (1900); A. S. Loevenhart and G. Peirce, Jl. Biol. Chem. **2**, 391 (1907); see also H. Davidsohn, Bio. Z. **49**, 249 (1913).

[4] Compare G. Senter, Ph. Ch. **44**, 257 (1903); **51**, 673 (1905).

on oxydases by hydrocyanic acid, which Warburg [1] has compared to the poisonous action of hydrocyanic acid with respect to the oxidation capacity of simple iron-containing carbon models. Not least worthy of mention are the extensive studies of Rona [2] and his coworkers with respect to the poisoning of lipatic enzymes by alkaloids, as a result of which the sensitivity towards quinine, for example, of a given lipase serves as a characteristic feature; thus, liver lipase has been found resistant while pancreatic lipase is strongly inhibited by quinine. The influence of associated materials upon this toxin sensitivity, to which Willstätter and Memmen[3] have called attention, has, meanwhile, not been sufficiently regarded and tested.

Anti-Enzymes.—The significance, finally, of the numerous observations in the literature with respect to the immunizing formation of *anti-enzymes* by the animal organism following the introduction of blood-similar or blood-dissimilar enzyme preparations cannot be definitely adjudged; in many cases it most probably may be ascribed to an adsorptive combination of the enzyme with the inhibition bodies which are formed.

[1] B. **58**, 1001 and particularly p. 1004 (1925).

[2] Bio. Z. **111**, 156; **118**, 185, 214, 232; **130**, 225; **134**, 108, 118; **141**, 222 (1923).

[3] H. **138**, 216 and particularly p. 241 (1924).

VI. ENZYMIC SPECIFICITY

The phenomena of enzymic specificity may be considered from two particular viewpoints, according to whether they concern the general course of the transformation of a substance which is accelerated by the enzyme., i.e., its reaction path, or whether they concern the susceptibility or velocity of the cleavage of various substances by the enzyme, i.e., its absolute and its relative specificity.

Specificity of Reaction Paths.—When we consider a variety of reaction paths according to which the enzymic transformation of a definite substrate takes place, we must distinguish between two different possible cases. That is, the splitting of a substrate by diffierent enzymes may result in different reaction products, or it may yield, even though by way of different intermediate substances, the given end products of the reaction. A characteristic example of the first case is furnished by the hydrolysis of the trisaccharide, raffinose, which, under the action of yeast saccharase, according to Neuberg,[1] results in the cleavage products, fructose, and melibiose, whereas, under the action of emulsin, on the other hand, cane sugar and galactose are formed. The researches of Abderhalden [2] have called attention to similar phenomena of reaction selectivity with peptid-splitting enzymes. The various types of fermentation of simple sugar, such as alcoholic or lactic acid fermentations, might also be mentioned here.

Characteristic of the second case, i.e., of specific reaction paths but identical reaction products, is the comparison of the hydrolysis of cane sugar by the saccharase of yeast and that of *aspergillus oryzae*. According to Kuhn,[3] the enzyme of yeast attaches itself to the fructose component of the cane sugar, whereas that of the mold fungus attaches itself to the glucose component of cane sugar.

[1] Bio. Z. **3**, 519 (1907).

[2] E. Abderhalden and A. H. Koelker, H. **54**, 363 (1908); **55**, 416 (1908); E. Abderhalden und C. Brahm, H. **57**, 342 (1908); E. Abderhalden, G. Caemmerer and L. Pincussohn, H. **59**, 293 (1909).

[3] H. **129**, 57 (1923).

Hence the inversion of cane sugar takes place by way of different intermediate reaction products; the two enzymes accordingly are to be regarded as different, specific catalyzers.[1] The opposing views of Euler and Josephson, which consist in the assumption of two different affinities of saccharases to cane sugar, are decisively refuted by more recent investigations of Kuhn and Münch [2] and by Kuhn and Grundherr,[3] that is, by exact affinity measurements and by specificity tests with various cane sugar derivatives. A further instance of the existence of different reaction paths with identical end products of hydrolysis is furnished by the significant investigation of Kuhn concerning starch cleavage by animal or vegetable amylase, which has resulted in the establishment of a distinction between α- and β-amylases.

Absolute and Relative Specificities.—Most of the current researches concerning enzymic specificity are devoted to the question as to which substrates are in general attacked by one and the same enzyme. One must discriminate here between an *absolute* and a *relative specificity*, according to whether the investigation shows that an enzymic catalyzer of two substrates is capable of transforming only one, or both of them, but with different velocities.

The phenomena of group specificity, which permit, for example, a discrimination between protein and fat-cleaving enzymes, are simple enough and are to be taken in the absolute sense. Moreover, the cases of absolute enzymic specificity are sufficiently numerous even in the hydrolysis of substrates of definite classes of substances. Thus, to mention only a few examples, the distinction between the specific action of the malt sugar and the cane-sugar splitting enzymes of yeast may be taken as absolute; furthermore, both enzymes have been successfully separated by preparative methods.[4] Then, too, our knowledge of the specificity of proteolytic enzymes, which had been difficult to visualize, has acquired a new basic principle through recent investigations; thus, Waldschmidt-Leitz and Harteneck [5] have shown that the

[1] See, however, H. v. Euler and K. Josephson, H. **132**, 301; **134**, 50; **136**, 62 (1924); **143**, 79; **149**, 71 (1925); R. Kuhn and H. Münch, H. **150**, 220 (1925); J. M. Nelson and R. S. Anderson, Jl. Biol. Chem. **69**, 443 (1926).

[2] H. **163**, 1 (1927).

[3] B. (*In press*).

[4] R. Willstätter und E. Bamann, H. **151**, 273 (1925–26).

[5] H. **149**, 203 (1925).

specific action of trypsin and erepsin are to be sharply differentiated from each other: whereas the first has been found specific for proteins themselves and their higher degradation products, the action of the latter concerns only substances of the type of simple peptides. Furthermore, the difference between pancreatic and intestinal erepsin as derived from the researches of Fischer and Abderhalden [1] cannot be maintained; the action of erepsin is specific for the hydrolysis of all simple peptides and for these alone.

The specific allocation of an enzyme to a substrate is, however, to be taken much more frequently in only a relative sense. It holds only in the more or less pronounced difference of velocity which concerns the transformation of the substrate. The phenomena of relative specificity are particularly significant and have been developed from many angles in the case of the fat and ester splitting enzymes. Thus, for example, according to the investigations of Willstätter and Memmen,[2] liver lipase is to be designated as an esterase, which is capable in only a limited degree of decomposing true fats, glycerine esters of higher fatty acids. Pancreas lipase, on the other hand, which is to be differentiated from it, represents a lipase which is most effective in hydrolyzing true fats. The multiplicity of specifically allocated lipolytic enzymes, particularly animal enzymes, is further augmented by the fact that the action of associated or added materials can be manifested in different directions according to the prevailing combination of enzyme and substrate.

Particularly carefully investigated examples of relative specificity, which depend on exact affinity measurements, have been carried out with the carbohydrate splitting enzymes. Thus the investigations of Kuhn [3] have shown, that the trisaccharide raffinose and cane sugar are both hydrolysed by one and the same enzyme, yeast saccharase. This conclusion is based upon the relations of the dissociation constants of the combinations of enzymes with cane sugar or raffinose, which are in agreement for all enzyme preparations investigated. Similarly, Willstätter, Kuhn and Sobotka [4] have found that, at times, both aliphatic and aromatic derivatives of α- or β-glucose are transformed by the same enzyme, namely, the maltase of yeast or the emulsin of bitter almonds.

[1] H. **46**, 52 (1905).
[2] H. **138**, 216 (1924).
[3] H. **125**, 28 and particularly p. 71 (1922–23).
[4] H. **129**, 33 (1923); **134**, 224 (1923–24).

Stereochemical Specificity.—Also the stereochemical specificity of enzymes, which has long been known, furnishes examples for all possible cases of differences of velocity, of absolute and relative specificity. It is conditioned by an asymmetry of the catalyst itself, whose reaction behavior with respect to the mirror-image, isomeric substrates has been strikingly characterized by Fischer with the lock and key simile. It has found a counterpart in the non-enzymic experimental examples of Bredig [1] and his school, e.g., in the asymmetric cleavage of racemic acids by optically inactive bases in the presence of optically active catalysts. The cleavage in these cases consists in the elimination of carbon dioxide from camphorcarboxylic acids.

In many cases of asymmetric enzymic hydrolysis the final outcome is realized by infinitely great velocity differences, accordingly, by an immeasurably low reaction velocity with the one isomer. This applies, for example, to the specificity of maltase or of emulsin, hence, exclusively α- or β-glucoside splitting enzymes, or likewise for the decomposition of racemic or optically active peptides by erepsin, which concerns only the substrates built up out of naturally occurring optical isomers. Characteristic examples of relative optical specificity are afforded, on the other hand, by the study of the enzymic ester cleavages, whose asymmetric course has been compared by Willstätter [2] in a series of cases.

Cases of relative specificity have also been described for the asymmetric course of enzymic syntheses; thus Rosenthaler [3] has shown that in the synthesis of mandelic acid nitrile, accelerated by emusin, the dextrorotatory antipodes are obtained predominantly, whereas Krieble [4] has proved that with the enzyme from peach leaves and from cherries the laevo-rotatory oxy-nitrile is obtained predominantly. The conclusion as to the existence of mirror-image, isomeric enzymes, advanced by Krieble, however, is in no wise to be regarded as certain, according to the results of investigations obtained, for example, with respect to the optical specificity of lipatic enzymes.

[1] B. **41**, 752 (1909); Ph. Ch. **73**, 25 (1910); Bio. Z. **46**, 7 (1912).

[2] H. **138**, 216 and particularly p. 245 (1924); **140**, 203 and particularly p. 221 (1924); **146**, 151 and particularly p. 156 (1925).

[3] Bio. Z. **14**, 238 (1908); **17**, 257 (1909).

[4] Am. Soc. **35**, 1643 (1913).

VII. DETAILED PROCEDURES IN PREPARATIVE ENZYME CHEMISTRY

1. QUANTITATIVE DETERMINATION

Activity, the Criterion of Concentration.—For a quantitative measurement of enzyme material, we still rely entirely upon a single characteristic property, their catalytic effectiveness. Even in the preparative experiments of Willstätter and his school, which in many cases have led to very extensive purification of individual enzymes, no positive procedure has yet been developed which would permit the description of an enzyme upon any other basis, such as a particular physical or chemical characteristic, for instance. Only in one exceptional case, that of vegetable peroxydase, have preparative experiments led to the recognition of another characteristic property of the enzyme itself, namely, the fact that it appears to possess color. However, even here, the characteristic color of the enzyme is recognizable and determinable in only the higher degrees of purity; in the plant raw material from which it is isolated, it is obscured by similarly colored, associated materials. Beginning with a higher enzyme concentration, the color of the solution runs parallel to its capacity of action. Even the numerous attempts of Euler to characterize the cane sugar splitting enzyme of yeast by something other than its catalytic properties, e.g., by the phosphorus or nitrogen content of the enzyme preparations, or by the analytical measurement of special decomposition products such as trytophane, have not yet led to positive results.

The idea of measuring enzymes quantitatively on the basis of their catalytic activity is indeed very old, but, remarkable as it may seem, its consistent application and particularly its transposition to the preparative method have not been attempted until recently. The older measurements of enzymic preparations were frequently conducted under dissimilar conditions and thus yielded non-comparable results. Accordingly, in only a few cases have

they permitted sufficiently correct expressions as to the quantity and the behavior of the enzymes. Moreover, it may be added that new considerations such as the dependence of the enzymic reaction velocity upon the concentration of the H-ions or upon that of the substrate, or also the influence of non-enzymic concomitant substances, which exert an activating or an inhibiting effect, could not as yet be taken into account. Credit is due to Willstätter for having recognized the importance of quantitative analysis in preparative as well as in descriptive enzyme chemistry, and for having developed the principles upon which a rational description of enzymes must be based. The applications of quantitative methods are comparable in a manner to the determination of dye yield and color intensity in the isolation of a natural dyestuff, and have been carried out by Willstätter along two lines, namely, by the quantitative control of preparative work with respect to the yield in relation to the source material and with respect to the enzymic concentration or the degree of purity in the solutions and preparations obtained. The observance and quantitative determination of enzymic yields, which evidently had to be undertaken, has led in many cases to important discoveries, both as to the applicability of the methods recommended in the older literature for obtaining crude enzyme preparations from vegetable and animal sources, and also with respect to the solubility relations of the enzymes and to their fixation in the cells themselves. It has likewise permitted an exact evaluation as to the success of the separation and purification processes, which is expressed in an increase in the degree of purity, i.e., in the relation between dry weight and enzymic effectiveness. The goal of preparative enzyme chemistry, that is, the continuance of purification until no further increase in the degree of purity can be attained, first found a positive basis with the introduction of quantitative controls.

Measurement Scales.—As a general measure for the quantity of an enzyme the term "enzyme unit" has been proposed by Willstätter and Kuhn.[1] This expression was first applied by Willstätter, Waldschmidt-Leitz, and Memmen [2] to a specific example, pancreas lipase, as the "lipase unit." The definition of such an enzyme unit depends upon the velocity with which the enzyme

[1] B. **56**, 509 (1922–23).

[2] H. **125**, 93 and particularly p. 115 (1922–23).

transforms the substrate under certain conditions, which differ with the individual ferments but are always expressed in the same manner among themselves. They may relate, for example, to the velocity constant of a mono-molecular reaction. As a measure for the enzymic concentration of a preparation, i.e., its degree of purity, we make use of the "enzyme values," e.g., the "lipase value," which are determined by the number of "enzyme units" in a given quantity by weight of the preparation; hence they increase in accordance with the degree of purity. These terms, which are clear and simple, can advantageously be used to replace the older measures, devised in a more circumstantial manner. The terminology recommended in general by Willstätter and Kuhn for the concentration of enzymic preparations may also be preferred to the expression proposed by Euler and Josephson [1] for the activity of the preparates. The latter expression in which

$$X(\text{enzymic activity}) = \frac{k \cdot g \text{ substrate}}{g \text{ enzyme preparation}}$$

is, as a matter of fact, not generally applicable. For one thing the course corresponds to only a limited number of enzymic reactions; the proportionality between enzyme quantity and the mono-molecular reaction constant in many cases is not valid. Furthermore, the dependence of the reaction velocity upon the substrate concentration, according to which $k \cdot g$ substrate would supposedly be regarded as constant, and upon which the proposed relation is based, does not hold generally. This disadvantage in the Euler definition is obviated in the expression used by Willstätter and Kuhn, since the concept of a "unit" especially developed for each enzyme takes into account the dependence of the enzymic activity upon all the factors which are recognized as influential and definitely fixes the conditions for measurement of an enzyme.

The definitions for the measurement units of enzymes, as given in the researches of Willstätter and his school in numerous examples, are based upon a consideration of the H-number of the medium. This H-number, of course, has a decisive bearing upon the enzymic reaction velocity and is usually selected optimally, and, further, is kept constant when possible throughout the dura-

[1] B. **56**, 1749 (1923).

tion of the measurement. Its influence, which has profoundly affected the measurements reported in the older literature, is maintained constant by the use of *p*H regulators. The definition of the enzyme units rests further upon the knowledge of the enzymic reaction process under the given conditions and upon the study of the relations which exist between the enzyme quantity and the extent of transformation. In simple cases, as for the validity of an equation of the first order, the magnitude chosen for the unit of the enzyme stands in an immediate relation to the velocity constants of the reaction; it is, for example, made equal to it. In other cases, its measurement relates to the time required for a definite transformation of the substrate or to a definite, measured transformation in a given time, the latter being derived from empiricially obtained time-transformation curves.

Another very important circumstance, which is capable of affecting the velocity of enzymic reactions and which has rarely been accorded adequate consideration in quantitative measurements, is that of the dependence of enzyme activity upon the presence of activating or inhibiting concomitant or addition substances. This condition is characteristic of many enzymes and is referable to both specific chemical phenomena and to nonspecific phenomena, the latter being, for example, those based on the adsorption effect. Such influences are removed by introducing a compensating activation or inhibition. That is, these influences are removed by adding activating or inhibiting substances in such proportions as to cancel the influence of the substances already present in the individual preparates. In this manner a comparable activity of the enzyme is assured. Thus, for the exact measurement of pancreatic trypsin, e.g., by the hydrolysis of high-molecular proteins, its complete activation by enterokinase is necessary; for the determination of animal amylase, the presence of a definite Cl-ion concentration is necessary; or for the testing of certain lipatic enzymes an equalization of the varying activation conditions by adding certain substances, characterized by their adsorption capacity, e.g., by adding proteins and lime soap, is necessary. This will be made evident in a few examples, which will be given below.

The measurements of the quantity and the concentration of many enzymes, e.g., those of yeast saccharase, have meanwhile, so far as they have been carried out, no strictly general validity.

According to Willstätter and Kuhn [1] they hold good only for the comparison of enzyme quantities which have been measured in preparates from the same source material, e.g., one and the same yeast race. The researches of Willstätter and Kuhn [2] have demonstrated that accessory and incompletely separated substances, perhaps disintegration products of the enzyme itself, which are associated with it in varying quantity, may affect its affinity to the substrate, and that therefore equal reaction velocities, measured under the same external conditions, are not in all cases based upon equal enzyme quantities. In order, then, to define with greater precision and to be able to compare enzyme preparates of different origins, the "enzyme units" and the "enzyme values" must be provided with the existing index of the dissociation constants of the enzyme-substrate compound. The latter represent their affinity to the substrate. For example, the comparison of yeast saccharase with unequal affinity is given by calculating the enzyme quantity obtained in the ordinary way for a saccharase of average affinity constant. However, for the comparison of ordinary preparative-analytic measurements such a reduction may be dispensed with.

Characteristic Examples.—The measurements selected for the unit of enzyme quantities will be illustrated subsequently by two characteristic examples.

Thus we designate as the unit of pancreatic amylase Am.-E.[3] the hundredfold of that enzyme quantity which gives the monomolecular reaction constant of 0.01 under the conditions of the experiment. These conditions involve action of the enzyme on 0.25 g. of soluble starch at 37° C and pH = 6.8 in a volume of 37 ccm. and after optimal and compensating activation by 1.0 ccm. of 0.2 n NaCl. The reaction constant, therefore, at the same time expresses the number of amylase units. The amylase value (Am.-W), which is the number of amylase units in 1 cg. of the substance, serves accordingly as a measure of the degree of enzyme purity.

The determination of the quantity of animal lipase can serve as a second example. Its activity, meanwhile, is regulated by the

[1] H. **125**, 1 (1922–23).

[2] H. **125**, 28 (1922–23).

[3] R. Willstätter, E. Waldschmidt-Leitz, and A. R. F. Hesse, H. **126**, 143 and particularly p. 156 (1922–23).

addition of non-specific regulators. Since the process of fat hydrolysis is not subject to any simple law, the basis for the determination of the lipase quantity is furnished by the empirical relations between enzyme quantities and the degree of saponification in a given length of time. A "lipase unit" L.E. [1] is defined as that quantity which will hydrolyse 24 per cent of 2.5 g. of olive oil in one hour under definite conditions, i.e., with compensating activation by means of $CaCl_2$ and albumin, an initial pH of 8.9 and a temperature of 30° C. This mode of designating the quantity and degree of purity of unknown natural substances, which hitherto have been measured only on the basis of their effectiveness, may also be applied to non-enzymic substances. Thus, according to Waldschmidt-Leitz [2] similar measures can be used for determining the specific trypsin activator, enterokinase, these measures being the "kinase unit" and the "kinase value," based upon the activating effect with respect to a given quantity of trypsin.

These arbitrarily chosen scales are valid only for the comparison of quantities of a given enzyme and, necessarily, do not permit any determination as to the quantity relations of different enzymes; nevertheless, the variability of the affinity of one and the same enzyme to accessory substances shows that even here the exact comparison of different enzyme quantities encounters difficulties. A positive basis for the quantitative comparison of different enzymes, as they occur in nature, will be obtained only by their preparative isolation in pure form.

2. OCCURRENCE AND FORMATION OF ENZYMES

Distribution and Occurrence.—Enzymes, as products of living cells and as catalysts of the most important chemical transformations, which constitute or condition the life process, are distributed throughout all cells of the plant and animal kingdom.

This is true for the one-celled and invertebrate organisms as well as the higher forms of plants and animals, even though in the latter their presence may be localized more recognizably and more significantly, that is, definite enzymic individuals are located

[1] R. Willstätter, E. Waldschmidt-Leitz, and F. R. Memmen, H. **125**, 93 and particularly p. 115 (1922–23).

[2] H. **132**, 181 and particularly p. 226 (1923–24).

in definite organs or secretions. This distinction in the lower organisms, e.g., in one-celled individuals, is not so easy to follow analytically because of the lesser differentiation of their separate organs.

In an introductory survey such as is given here, it is neither possible nor necessary to give a complete compendium of all the data thus far reported as to the presence of enzymic action in the separately examined organisms or in their organs or secretions. At the same time the differentiation or identification of many enzymes of similar action but of different occurrence has been exactly determined in only a minority of cases. Accordingly, Table XXII, in which a number of enzymes are described as to their occurrence and action, represents a selection of only the most important and most carefully investigated enzymes.

Exo- and Endo-enzymes.—The supposedly essential distinction, which the older school of enzyme research raised between the so-called exo-enzymes and the endo-enzymes, has not been confirmed in the older sense by the results of the newer preparative enzyme chemistry. (The so-called exo-enzymes were those freely dissolved or secreted in solution and the endo-enzymes were held to be insolubly attached within the cell and inseparable from living protoplasm.) It has developed that it is impossible to draw any fixed line of demarcation between extra-cellular and intra-cellular enzymes based on their solubility relations in their natural milieu, even though many enzymes, whose work is accomplished within the cell itself, are not contained free in solution, but are somehow fixed by adsorptive union to insoluble carriers and cannot be extracted from the cell by the mere use of solvents.

Liberation of the Enzymes.—Attention was first drawn to these phenomena by the classical researches of Buchner,[1] in which he demonstrated the liberation of the fermentative enzyme complexes upon mechanical destruction of the yeast cell structure. Our knowledge as to the solubility or the manner of combination of the enzymes in their naturally occurring state has been profoundly broadened and extended by subsequent researches; these have also become of practical importance with regard to preparative isolations. Thus, for example, the researches by Willstätter and Racke [2] have shown that a solution of the cane-sugar splitting

[1] B. **30**, 117 (1897); E. Buchner, H. Buchner and M. Hahn, Die Zymasegärung. München, 1903.

[2] A. **425**, 1 (1920–21); **427**, 111 (1921–22).

TABLE XXII

CLASSIFICATION AND OCCURRENCE OF THE MOST IMPORTANT ENZYMES

Group	Member	Occurrence (by Illustrations)	Substrate (by Illustrations)
Esterases	Phytolipase	Oil containing seeds (e.g., ricinus seeds)	Fats
	Animal esterase	Liver	Simple esters
	Animal lipase	Pancreas, Stomach	Fats
	Chlorophyllase	Green leaves	Chlorophyll
	Tannase	Aspergillus niger	Tannins
	Phosphatase	Yeast	Phosphoric acid ester
	Sulfatase	Aspergillus oryzae	Sulfuric acid ester
Proteases	Pepsin (lab)	Stomach	Genuine proteins (casein)
	Trypsin	Pancreas	Genuine proteins
	Erepsin	Pancreas, intestine, yeast, animal tissue	Peptides
	Papain	Melon tree	Genuine proteins
Amino-acylases	Urease	Soya bean	Urea
	Histozym	Kidney	Acyl amino acids
	Arginase	Liver	Arginin
Carbo-hydrases	Amylase	Ptyalin, pancreas Malt, *Asperg. oryzae*	Starch
	Saccharase	Yeast, *Asperg. oryzae*, intestine	Cane sugar
	Maltase (α-Glucosidase)	Yeast	Maltose, α-glucosides
	β-Glucosidase	Bitter almonds	β-Glucosides
	Lactase	Yeast	Lactose
	Nuclease	Intestine	Nucleic acids
Oxidation enzymes	Peroxydase	Roots, embryos	Phenols + H_2O_2
	Tyrosinase	Potato	Tyrosin
	Uricase	Liver	Uric acid
Catalases	Catalase	Yeast, Liver	Hydrogen peroxide
Fermentation enzymes	Zymase complex	Yeast	Grape sugar
	Carboxylase	Yeast	Carboxylic acids
	Carboligase	Yeast	Aldehydes

enzyme cannot be prepared from yeast simply by crushing and pressing or simply by lixiviation of the cell substance; the extract from such a process will contain only a small fraction of the total enzyme content. Although it is true that the saccharase of yeast is free, water-soluble and not combined with insoluble constituents in the yeast cell, yet the enzyme cannot be extracted from the latter simply by the action of a solvent; it is successfully extracted only after complete mechanical destruction of the cell structure, which cannot be accomplished merely by comminution and tearing of the cell membrane. In other words, the enzyme finds itself enclosed in the cell by a protective layer, which may also be destroyed by enzymic degradation, a process which can be distinguished from autolysis, the general dissolution of the cell. This is true since, after the yeast cells had been killed, the saccharase was successfully transferred into solution by the action of diastatic ferments only after the proteins had been removed by proteolytic enzymes. In still other cases, quantitative analysis of the yields in enzymic extracts have shown that the enzymes are held fast in the cells in the form of adsorption combinations, which are not easily distinguished from chemical combinations; in these cases the adsorption affinities have been overcome by mild chemical agents and the yields considerably increased thereby. Thus, emulsin [1] from bitter almonds and peroxydase [2] from cereal sprouts were carried over into the solution in very small quantities by means of water, while very dilute alkalies, on the other hand, greatly increased the yield. According to Willstätter,[3] these observations may also contribute toward explaining the manner of combination of other high-molecular substances. Thus the discoveries concerning the solubility behavior of chlorophyll in leaves or the coloring matter in flowers indicate that these bodies, e.g., the chlorophyll in the chloroplasts, are not freely dissolved, but are chiefly present in adsorptive combination with non-soluble carriers, through which their solubility behavior is altered. The destruction of these adsorption combinations is effected by relatively small quantities of alkaline or acid reagents.

Solubility Gradations.—All gradations of stability are recognizable in the union of enzymes with other cell-constituents, as

[1] R. Willstätter and W. Csanyi, H. **117**, 172 (1921).
[2] R. Willstätter and A. Pollinger, unpublished.
[3] B. **55**, 3601 (1922).

well as in their solubility behavior within their natural media, and correspondingly in their susceptibility to extraction. Thus, the enzymes found in plant and animal organisms, e.g., in the digestive juices, the typical extra-cellular enzymes according to the older school, are freely dissolved in these secretions. Also, they can often be readily transferred into solution before their secretion out of the fresh or dried cell-substance, as has been shown in experiments upon the extraction of the three most important enzymes of the pancreatic gland, lipase, trypsin and amylase.

As characteristic examples of the influencing of the solubility of an enzyme, either by mechanical covering or by adsorptive union with insoluble cell-substances, we may mention the cases already cited of yeast saccharase on the one hand and of emulsin or of peroxydase on the other. Also the discovery concerning the solubility behavior of the starch-splitting enzymes of ungerminated cereals, as described by Baker and Hulton [1] are probably to be classified in this group. The same thing is true of the solubility of an interesting enzyme, discovered by Schmiedeberg; [2] the action of this enzyme, histozyme, which has not yet been extensively studied, consists in the synthesis and hydrolysis of acylated amino acids, e.g., of benzoyl glycocoll. In the same regard, Willstätter, Waldschmidt-Leitz, and Waldschmidt-Graser [3] have been able to show that the extractability of the enzyme from the crude pancreas, as well as from the dried gland, varied with the type of animal under observation; the histozyme of the dog kidney is readily dissolved in aqueous solutions, that of the swine kidney only incompletely, that of the horse kidney not at all.

These unequal solubilities of the enzymes must be attributed to an adsorptive union with insoluble gland-substance of varying stability, and not to a difference in the enzymic individuals themselves, even though a decomposition of the adsorption-compound has not yet been accomplished. The only enzymes whose complete insolubility may be regarded as definitely proved belong to the group of plant lipases, which are found, for example, in the seeds of oil-bearing plants. Thus it is evident from a study of the ricinus lipase, made by Willstätter and Waldschmidt-Leitz,[4] that this enzyme is insoluble in water as well as in glycerine, and

[1] Soc. **121**, 1929 (1922).
[2] Virch. Arch. **14**, 288 (1881).
[3] *Yet unpublished.*
[4] H. **134**, 161 (1924).

is indeed highly unstable with respect to the action of glycerine, water or electrolyte solutions. Even in the germination of plant seeds, where its action takes place, the enzyme is not mobilized and carried over in soluble form, but the glycerides for saponification, suspended in the cytoplasm of the seed, are presented to it in the cell itself. This difference of solubility between the fat-splitting enzymes in plant seeds and the lipase, for example, of the animal digestive tract corresponds to the locally prevailing conditions for fat-cleavage.

In order for the soluble pancreas lipase to function it needs to establish a certain adsorption state which will facilitate contact with the insoluble substrate (see Chapter V). The activators required for this, such as proteins and gall salts, are found in the locus of the reaction, in the intestine; the enzyme is brought into reaction with the fats by means of these activators, with the formation of complex adsorbates. The plant lipase on the other hand, which enters into the reaction only within the seed-cell, is either attached by adsorption to an insoluble bearer of protein nature or else the protein substance is, as a constituent of the lipase molecule itself, the colloidal bearer of the enzymically active group; in germinating it undergoes an alteration by which it acquires the power to exert its effect under the conditions presented in the cell, i.e., in a neutral reaction. This alteration, which may likewise be excited artificially by the action of proteolytic enzymes, concerns the protein portion of the enzyme molecules; however, its solubility is not affected thereby, but only the affinity relations of its chemically active group.

Enzyme Formation and Secretions in Animal Organisms.— Because of the limited knowledge and insufficient observance of the solubility states of the enzymes, many communications in the older literature with regard to the formation of enzymes in living and dead cells are totally discredited today. Only a few of the older observations which have been carried out on the formation of soluble, freely secreted enzymes, may yet be regarded as valid to any extent. Among those observations whose significance can be accepted today belong the remarkable findings of Pawlow [1] concerning the adaptation of the ferment formation and secretion in animal organisms to the type of food being administered. From Table XXIII, taken from Pawlow's researches, it can be seen

[1] Die Arbeit der Verdauungsdrüsen, p. 48 ff. Wiesbaden, 1898.

that the relative content of the pancreatic secretion in the three most important digestive enzymes (protein-, fat- and starch-splitting enzymes) corresponds in a marked degree to the composition of the food administered. With an increase in a given constituent of the food the gland responds by an increase in the production and separation of the enzyme required for the conversion of that constituent. The data in the table, which, it must be admitted, do not satisfy modern requirements for quantitative determinations, and which have been disputed in a research made by Wohlgemuth,[1] are of considerable qualitative significance, even though they do not have a strictly quantitative value. Moreover, these results correspond to those obtained in the more recent observations with respect to the increased enzyme formation in one-celled organisms.

TABLE XXIII

Relation between Food Administered and Enzyme Content of the Pancreatic Secretions

(*According to Pawlow*)

Food	Quantity of Secretion, Ccm.	Trypsin	Amylase	Lipase
600 ccm. milk.....	48	1085	432	4334
250 g. bread.....	151	1978	1601	800
160 g. meat.....	144	1502	648	3600

Enzyme Formation in One-celled Organisms.—In 1882 Wortmann [2] published some noteworthy observations as to the dependence of enzyme formation upon the food substrate with one-celled organisms. These showed that bacteria are capable of forming starch-cleaving enzymes only when they have at their disposal no other utilizable carbohydrate source besides starch. Meanwhile the efforts of Wortmann to prove that in similar manner there is an influence exerted by the composition of the food upon the power of the yeast to form the cane-sugar splitting enzyme did not yield positive results; the quantitative principles

[1] II. Berliner klin. Wochenschr., 1907, p. 47. [2] H. **6**, 287 (1882).

of the enzymic method had not yet been sufficiently developed. The same objection is to be raised against a subsequent research by A. Fernbach,[1] in which the enzyme content was measured not in the yeast itself, but only after a circumstantial and uncontrollable autolysis; also no definite conclusions can be drawn from the latter work by Went [2] with respect to the influence of the food upon enzyme formation in monilia sitophila. Systematic investigations as to the formation of saccharase in yeast were not further undertaken until the work by Euler[3] and his collaborators in 1910; these results were confirmed and amplified by the valuable observations of Meisenheimer, St. Gambarjan and Semper.[4]

The researches of Euler's laboratory are based on the exact definition of the inversion power of living yeast cells, which is given by the equation

$$\text{Inversion} = \frac{k \cdot g \text{ sugar}}{\text{Number of cells}}$$

or else, when the number of yeast cells is not known, by the corresponding expression

$$I_f \text{ (Inversion capacity)} = \frac{k \cdot g \text{ sugar}}{g \text{ dry substance}}$$

which relates the catalytic activity to the quantity of dry substance present. Within the limits maintained, this magnitude is only slightly dependent on the amounts of yeast and cane sugar employed. It has been found that the saccharase content of two yeast races, a Stockholm bottom yeast and top yeast, showed a remarkable constancy for nine or three years, respectively; it corresponded to an average value for I_f of 16 or 9^{-2}. An increase of the enzyme content could be obtained in either of two ways: first (not very appreciable, to be sure), by providing a highly nitrogenous food-medium, e.g., peptone, or second (and in much larger measure) by growing the fungus on a nutrient solution of a high cane-sugar concentration. In this case the inversion capa-

[1] Ann. Inst. Pasteur **4**, 641 (1890).

[2] Jahrb. wiss. Botanik **36**, 311 (1901).

[3] Arkiv. f. Kemi **3**, nr. 34 (1910); H. **70**, 279 (1910–11); **76**, 388; **78**, 246; **79**, 274; (1912); **84**, 97 (1913); **88**, 430; **89**, 272 (1914); Bio. Z. **58**, 467; **67**, 203 (1914); H. **97**, 286 (1916); Bio. Z. **85**, 406 (1917–18); H. **106**, 201 (1919); **109**, 65 (1920).

[4] Bio. Z. **54**, 122 (1913); **67**, 364 (1914).

city of the yeast increased markedly with the duration of the cultivation procedure and this up to a maximal value; by transferring yeast thus enriched to a fresh nutrient solution of equivalent value as the first, still further increases were obtained in the enzyme content, whose absolute amounts, however, became steadily smaller. By this method of yeast breeding in high concentrations of sugar, Euler and Meisenheimer each obtained an enrichment of the saccharase in the fungus, up to an approximately 10-fold amount in the best examples; the time value of the cell material, which is inversely proportional to the enzymic concentration, fell, for example, from 480 to 42.

Further Enrichment of Invertase in Yeasts.—The enzyme increase observed in these examples, meanwhile, has been repeated and considerably improved upon by the experiments of Willstätter, Lowry, Jr., and Schneider.[1] These experiments have led to the finding that the decisive condition for obtaining the most favorable formation in yeast is constituted by the mode and manner of the fermentation procedure. Breeding by fermentation in a concentrated sugar solution is replaced by carrying out the fermentation in a solution of minimal sugar concentration. The fact that more saccharase is found through a smaller sugar addition than with a larger addition is to be ascribed to the condition of excitation in the yeast. Providing fermentable sugar to the fungus appears to exert a stimulation, which causes the fungus to enter a state of readiness for fermentation. Hence the production of the normal enzymes is found to be less dependent on the state of nutrition than on the release of definite specific stimuli. The procedure adopted by Willstätter for continuously providing very small amounts of sugar, e.g., by slowly dripping a sugar solution into the well agitated fermenting material, yields reproduceable values of the enzyme increase, even though the behavior of the living fungus varies from case to case according to its physiological condition. This latter is influenced, of course, by its previous history. In this manner, high enzymic concentrations are obtained in a few hours, their time values in the best cases being between 15 and 22, which is 16 times better than in the original material. It is a noteworthy fact that such an increase of the saccharase content is also observed in the ordinary autolysis of the fungus; the saccharase formation is not post-mortal in this

[1] H. **146**, 158 (1925).

case as was formerly supposed, but precedes the killing of the cell, coupled with the fermentation of the carbohydrate content.

Comparison between the growth of the saccharase content in the yeast and alteration of other enzymic yields has led to valuable conclusions regarding enzyme formation in living fungus; the saccharase appears to assume an independent position in the enzymic equipment of the yeast. It has been proven that no increase in the fermentative power of the yeast follows the increase in saccharase and that the other enzymes—those susceptible to measurement, as maltase or the yeast proteases—remain unchanged in quantity. "Hence, the relation of the saccharase to the enzymes which accompany it, is increased almost in equal degree with its enrichment." Its formation depends upon the specific physiological excitation state of the fungus.

Adaptation of Yeast to Various Substrates.—It is of interest in this connection to mention briefly those observations concerning the adaptation of yeast to certain sugars which are not fermentable under the ordinary conditions. There are numerous statements in the literature to the effect that most yeasts, incapable of fermenting galactose, may acquire the ability for such fermentations by being bred in solutions of this substrate. The fermentative capacity for galactose may even surpass, in the process of habituation, that measured for glucose, if the fermentation velocity of these two substrates is considered singly. The relation of the fermentation velocities as determined in a mixture of substrates, according to Willstätter and Sobotka,[1] differs considerably from the velocity relations of the separate fermentations, the tendency being toward a more rapid glucose fermentation. This increase in the fermentative capacity for galactose was formerly ascribed to a biological adaptation connected with the life of the yeast cells. However, Euler and Nilsson[2] have shown that a sterile dry yeast, in the same degree as fresh yeast, likewise acquires the power of fermenting galactose, by pre-treatment with the sugar. Through further experiments by Euler and Lövgren [3] it has been shown that the newly acquired fermentative capacity of the yeast suffers no retrogression after its return to the natural nutritive medium, glucose, even over long periods of time. Hence, the adaptation of the yeast to its fermentation substrate is not to be compared to that of the enzyme

[1] H. **126**, 176 (1922). [2] H. **143**, 89 (1925). [3] H. **146**, 44 (1925).

increase observed for saccharase; it is rather to be ascribed to a purely chemical or enzymic transformation of the zymase complex.

3. TECHNIQUE OF PREPARATION AND PURIFICATION

The methods employed for isolating enzymes from the plant or animal cell-substance, so far as the enzymes are not to be obtained in a freely dissolved state in the form of secretions, are modified according to the varying solubilities of the enzymes. Completely insoluble enzymes, such as the lipase of plant seeds, require a preparative method for purification which is essentially different from that which has been evolved in many cases for enzymic individuals in solution; they will be treated separately, therefore, at the end of this section.

Solution after Destruction of Cell-structure.—In transferring an enzyme from a plant or animal source to an aqueous infusion, e.g., from a plant root, a fungus culture or an animal gland tissue, several possible procedures are open depending on the nature of the material. The cell structure can be opened up by severe mechanical fragmentation of the cell membrane in order to separate the soluble cell contents and the enzyme in the form of an expressed juice from the insoluble cell constituents, as has been carried out by Buchner in his well-known research upon the isolation of the zymase complex from yeast. It has already been pointed out, in the instance of yeast saccharase, that only a fraction of the enzyme is recovered in the expressed juice by this method, the amount depending on the condition of the enzyme in the cell. At times a very effective procedure in expressing the material of the fragmented cell has been to fix the active enzyme on some insoluble cell substance and then to rinse out the enzymically inactive components. Thus, peroxydase in the plant root may be precipitated on the plant fibers by mild acid action and may then be freed from a part of the ballast by dialysis, without thereby losing any of the enzyme; the enzyme goes over into the expressed juice only after the acid has been neutralized with dilute alkali.

Autolysis.—Another process, which is frequently used and which permits the isolation of the enzymes in satisfactory amounts if certain precautionary measures are taken, is based upon the automatic dissolution of the cell—autolysis. After the death of the cells, possibly by cell poisons, a greater part of the cell con-

tents are carried over into solution by the enzymic degradation processes which are now uninhibited. The enzymic concentration of such autolysates will be found less favorable than in the expressed juices, because of their greater content of the enzymically solubilized concomitant materials. Meanwhile the more extensive disintegration of the cell-substance may be of advantage in many cases for the further purification of the enzyme, for example, with precipitation or adsorption agents. Also, fractional evacuation of the dead cell-substance is accomplished by the introduction of definite enzymic disintegration processes; thus the saccharase of yeast, which is not emptied out with the decomposition of the greater part of yeast albuminous materials by trypsin, may be liberated in greater purity by the subsequent action of diastatic enzymes.[1] In the release of enzymes by autolytic processes, care must be taken, however, that the enzymes themselves are not destroyed by alterations of the medium. Thus Willstätter, Oppenheimer and Steibelt,[2] have shown that the maltase of yeast, which is very sensitive towards acids, is for the most part destroyed in the ordinary autolysis of the fungus by the acids which are formed and that it is only possible to separate maltase in any large degree from the cell-substance when care is taken to neutralize the acids formed.

Drying and Subsequent Extraction.—In other cases, and particularly when working with animal source materials, it is better to dry the finely divided cell-substance before its lixiviation. The advantage of such a procedure is that a part of the cell protein is denatured and that, as a result, the enzymes are usually obtained in greater concentration in the extracts obtained by treating the dried materials with solvents, than when the fresh tissues are employed. It is only necessary that care be taken that the action of the dehydrating medium does not injure the enzymic activity. Thus it has been shown that pancreas lipase is appreciably destroyed when the gland tissue is dried by means of alcohol.[3] On the other hand, dehydration and de-fatting of the gland with acetone and with ether, which are generally applicable, are harmless to the lipase as well as to the other enzymes.

[1] R. Willstätter and F. Racke, A. **427**, 111 (1921).

[2] H. **110** 232; **111**, 157 (1920).

[3] R. Willstätter and E. Waldschmidt-Leitz, H. **125**, 132 and particularly p. 148 (1922-23).

Extraction of the enzyme from the dried material is effected by an aqueous medium or by glycerine, the fitness of which as a solvent for animal enzymes was first pointed out by Wittich; these are ordinarily more stable in a solution containing glycerine than in a purely aqueous medium. In certain cases extraction is accomplished by the action of dilute acids or of dilute alkalies, when the dissolving of the enzyme is restrained by its adsorptive binding. This is true of the emulsin of bitter almonds, which cannot be dissolved from the dry kernels until they are subjected to the mild action of an alkali.

Dialysis.—Among the methods employed in the earlier investigations, chiefly for the further purification of enzymes from their crude solutions, the use of dialysis procedures may be mentioned. These permit a separation of the electrolytes and of the lower molecular concomitant substances, while the animal membranes will not allow the enzyme itself to pass. The increase in the degree of purity attainable by dialysis is not very great; its importance for preparative enzyme chemistry rests rather upon its use for the more extensively pre-purified enzyme solutions, which may thus be freed from associated or adsorbed ions, or also from organic media, such as glycerine.

Precipitation.—Other procedures may be used to separate enzymes from their solutions; for this purpose organic solvents have been used chiefly, such as alcohol or acetone; the salting out effect with ammonium sulfate has also been used, and particularly the action of heavy metal salts and of protein-precipitating agents have been employed; the protein-precipitating agents most often employed are salts of mercury, lead or uranium or tannic acid. But in most cases these precipitations when observed are to be ascribed, not to the enzyme itself, but to its associated materials; they depend largely upon the varying, more adventitious composition of the enzyme solutions. It is true that saccharase may be separated from some of its solutions, as has been proved, by lead acetate or by uranyl acetate in precipitation reactions, but the enzyme is merely adsorbed by these precipitations, which are to be ascribed to the concomitant protein substance. Only the precipitation of plant peroxydase by tannin, described by Willstätter and Pollinger,[1] appears to be a reaction of the enzyme itself. It may be added that the sensibility of many enzymes, above all,

[1] A. **430**, 369 (1922–23).

those of the animal organism, with respect to the alteration of condition upon their precipitation from aqueous solution, frequently results in appreciable loss in enzymic activity. Thus pancreas lipase dissolved in an aqueous glycerine-containing medium is almost entirely destroyed by the action of alcohol or ether, even in amounts which do not suffice for its precipitation; also, serious losses of activity occur in the precipitation from its solutions by heavy metal salts.

Adsorption of Enzymes.—For the isolation of enzymes from their natural mixtures with large amounts of foreign, associated materials, principally proteins and carbohydrates, no points of attack by chemical means are presented so far as we now know. Apart from the reaction potentialities of their specifically active group they must be regarded as chemically indifferent substances. Furthermore, observations of their precipitability relate, as just explained, not to the enzymic individuals themselves but to their associations with concomitant substances. The isolation of the enzymes is rendered still more difficult by their unsatisfactory solubility behavior; their insolubility in many organic solvents, especially in those immiscible with water, affects their separation from foreign materials. Hence a method, such as the one that has been successful in the isolation and purification of animal or vegetable coloring matters, is but poorly adapted to the purification of enzymes. "There exists but a single method for the isolation of enzymes which is versatile, adaptable and capable of development, namely, the employment of the adsorption processes based upon small affinity relations or upon residual affinities."[1] It was by this means that success was first attained in securing a higher degree of purity with a number of enzymes.

The purification of enzymes by adsorption, according to the experiments of Willstätter and his colleagues, is confronted with three particularly important problems. One such task consists in making the adsorption of an enzyme selective as far as possible. Adsorbents, which under ordinary conditions take up a considerable amount of associated and intermingled substances along with the enzyme, will be of essential importance for purification purposes only under such conditions as those in which its adsorption is found to be specific to the highest possible degree. The smaller the amount of the adsorbent required to take up the enzyme, the

[1] R. Willstätter and E. Waldschmidt-Leitz, H. **125** (1922–23).

higher may be expected the degree of enzymic concentration. The determination of " adsorption curves " has proved serviceable in evaluating the selective action of an adsorbent. According to Kraut and Wenzel [1] these curves reproduce the relations between the concentration of the enzyme in the adsorbate and that in the adsorption mother liquor. They permit a recognition as to those conditions under which an enzyme, in its existing degree of purity, reacts most specifically with the adsorbent. The conditions of adsorption may be varied according to a change in acidity, according to an alteration of the initial concentration, e.g., by dilution of the solution, or according to a pre-adsorption of enzymically inactive materials as elucidated in Chapter III. These curves, in particular, permit a conception as to the existing degree of purity of the enzyme, according to whether they exhibit the appearance of an integrally adsorptive system consisting of substances resembling each other in adsorption behavior or whether they represent a mixture of substances with very unlike adsorption affinities.

A second problem that confronts the adsorption methods and that has already been carried out successfully in a few examples consists in the separation of certain enzymes from other naturally associated enzymes. Thus, the secretions and extracts of the pancreas constitute a mixture of four specially important enzymic substances; fat-, starch-, protein-, and peptide-splitting enzymes. As a preliminary for the investigation of one of these pancreatic enzymes, the separation of the other accompanying enzymes must be considered and the correspondingly suitable adsorption methods must be worked out.

A third problem, practically unsolved as yet, is presented by the separation of the enzymically inactive pre-stages and decomposition products of the enzyme, which stand nearest the latter in adsorption behavior. It is still a matter of uncertainty as to the extent to which the adsorptive medium is able to choose between the enzymes and those substances which differ from them only by the lack of the specifically active group. Only in the example of a substance which is non-enzymic, yet closely related to the enzyme in other points of behavior, i.e., in entero-kinase, the specific trypsin activator in the intestinal membrane, has it been made certainly evident that its adsorption behavior toward the

[1] H. **133**, 1 (1923–24); **142**, 71 (1924–25).

adsorbent, alumina, coincides with that of its pre-stage, "prokinase," which is formed in the pancreas.[1]

Early Attempts with Adsorption Methods.—The first studies of the adsorption of enzymes and of their separation from their natural mixtures by adsorption date from the years 1861 and 1863. Brücke [2] succeeded in "binding pepsin mechanically to small solid bodies," e.g., to calcium phosphate, sulfur, or cholesterin, and in again freeing the enzyme from its adsorbate by an alteration of the reaction or also by dissolving away the adsorbent itself. Danilewsky [3] soon afterward, by means of adsorption media, undertook the separation of the three most important enzymic functions of the pancreatic juice, which he attributed to specific carriers. By adsorption on collodion, he was able to obtain amylase-free trypsin from the lipolytically inactive secretion, while the amylase, still associated with a part of the trypsin, remained in the adsorption mother liquor. Cohnheim [4] in the same year reported the separation of the starch-splitting enzyme by adsorption on calcium phosphate. In another instance, Hammersten [5] attempted a separation of enzymes by adsorption. He described a process for obtaining pepsin-free solutions of the labic enzyme from infusions of the mucous membrane of the stomach by means of fractional precipitation with magnesium carbonate or a solution of lead acetate. But these findings have not been verified; according to subsequent researches by Pawlow and Parastschuk [6] and by Hammersten [7] pepsin-free solutions of lab are not obtained in this way; rather, the solutions contain inactive pepsin.

Willstatter's Separation of Pancreatic Enzymes.—The task of obtaining a separation of enzymes by adsorption was first taken up in an extensive way in the researches of Willstätter; they were based upon the delicate gradations which the enzymes exhibit in their acid and basic properties. Willstätter and Waldschmidt-

[1] E. Waldschmidt-Leitz, and A. Harteneck, H. **149**, 221 (1925).

[2] Sitzungsber. math.-naturw. Klasse d. k. Akademie der Wissensch. Wien **43**, 601 (1861).

[3] Virch. Arch. **25**, 279 (1862).

[4] Virch. Arch. **28**, 241 (1863).

[5] R. Malys Jahresberichte über die Fortschritte der Tierchemie, 2. Band für 1872, p. 118 (1874).

[6] H. **42**, 415 (1904).

[7] H. **56**, 18 (1908).

Leitz,[1] have determined the acid and basic properties of the three most important pancreatic enzymes, lipase, amylase and trypsin, and have laid the foundation for their separation by adsorption on the basic and acid adsorbents, alumina-gel and kaolin. Pancreatic lipase, which is strongly influenced in its reaction system by unspecific adsorption relations, is correspondingly very easily adsorbed and in this respect is only slightly dependent on associated materials. Thus, it is readily adsorbed by basic as well as by acid adsorbents, and by alumina or kaolin, but its acid properties are more strongly pronounced than those of amylase and trypsin; hence it can be entirely freed from the accompanying enzymes by adsorption with alumina. Even when the presence of certain concomitant substances, which act as "coadsorbents" has the effect of carrying over with it into the alumina adsorbate a part of the amylase and trypsin, a simple repetition of the adsorption procedure suffices to complete the separation. Trypsin and amylase remain in the mother liquors of the alumina adsorption, while elution of the adsorbates yields the lipatic components.

Pancreas amylase is indifferent to both basic and acid adsorption media; when its adsorption is observed, it is to be ascribed merely to the influence of concomitant substances. In a purer condition it is not taken up from an aqueous solution either by aluminum hydroxides or by kaolin, hence, it is lacking even in basic properties. Upon this fact is based its separation from trypsin, which is adsorbed with relative facility by electronegative media. Amylase may be successfully freed from trypsin by repeated treatment with kaolin in acid solutions, and in many cases even in neutral solutions, the trypsin being recovered in the elutions of the kaolin adsorbate with dilute alkali. Thus, the enzymic mixture of the three most important enzymes in the pancreas extracts is separated by successive adsorptions with alumina and with kaolin.

The separation of the three pancreatic enzymes is completed and extended by the investigation and separation of pancreas erepsin, the peptide-splitting enzyme of the gland, which Waldschmidt-Leitz and Harteneck[2] have differentiated from the tryptic glandular enzyme on the basis of its specific action; it differs from this also in its adsorption affinities—its acid proper-

[1] H. **125**, 132 and particularly p. 142 (1922–23).
[2] H. **147**, 286; **149**, 203 (1925).

ties are more strongly marked. Thus the adsorption by alumina and the elution of the adsorbates obtained, as in the crude acidified extracts, leads—upon repeated application—to a complete separation of both proteases. The trypsin remains in excellent yield and free from ereptic action in the residuary solution of the last adsorption treatment, while elution of the alumina adsorbates yields integrally the ereptic components. It is very remarkable that both these enzymes, which belong to the same class of enzymic substances, should display such a distinct difference in their adsorption properties. Their qualitatively different behavior toward alumina is to be attributed to a reaction of the enzyme itself, and not to chance associations with concomitant substances. The same behavior is evidenced, for example, in their separation from an entirely different source, the extracts of the intestinal mucous membrane;[1] further, a separation of the protein- and peptide-splitting enzymes of yeast has been accomplished in a similar manner.[2]

Recognition and Differentiation of Adsorption Agents.—Our knowledge of the separation by adsorption of two enzymes closely related in their specificity, was very recently enlarged by a still further example. Willstätter and Bamann [3] have shown that both carbohydrases contained in the crude autolysates of yeast may be quantitatively separated by the selective adsorption of an alumina gel; these carbohydrases are the cane-sugar splitting and the malt-sugar splitting enzymes. It was found herein that the simple observation, according to which the selective action of an adsorbent is to be attributed to its acid or basic properties, is no longer adequate; that is, the most favorable selectivity is to be observed in an alumina gel which no longer reacts perceptibly with dilute acids or alkalies.[4] Thus it appears that for the fitness of an adsorbent for the separation of enzymic individuals, certain affinity relations are decisive which have not yet been precisely defined with respect to their electro-chemical and colloidal chemical nature. Data concerning the separation of enzymes will be extended in every case by a more profound knowledge of the adsorption media especially fitted therefor.

[1] Waldschmidt-Leitz and A. Schäffner, H. **151**, 31 (1925–26).
[2] Willstätter and Grassmann, H. **153**, 250 (1926).
[3] H. **151**, 273 (1925–26).
[4] See R. Willstätter, H. Kraut and O. Erbacher, B. **58**, 2458 (1925).

Marked progress has been made in recent years with respect to the chemical characterization of important adsorption media, by the researches of Willstätter and Kraut, " Uber Hydrate und Hydrogele." [1] Thus it has been shown that according to the method of preparing the adsorbent alumina, including the conditions of its precipitation, various sorts of alumina hydrate are obtained which differ remarkably in adsorption capacity and in selective action. The difference, which cannot be explained by a different dispersity—the varying degrees of division of the colloidal particles—may be ascribed to the existence of different chemical compounds, which may be recognized by their content of chemically combined water as well as by their behavior toward dilute acids and alkalies. A preliminary investigation [2] has led to the definition of a distinction between four different types of aluminum hydroxide whose formation is determined by the more intimate conditions of their precipitation:

Aluminum hydroxide *A*, precipitated with an excess of ammonia and boiled a long time—plastic;

Aluminum hydroxide *B*, precipitated in the same way as *A*, but without prolonged heating—plastic;

Aluminum hydroxide *C*, precipitated with dilute ammonia, without long heating — fine-grained, pulverent;

Aluminum hydroxide *D*, precipitated from aluminate solution with carbon dioxide, coarsely pulverent.

The improvement in the preparative procedures for the production of the adsorbents and the extension of their analytical investigation make it possible, in the newer works,[3] to characterize some of the preparates as definite chemical individuals, as definite hydrates of aluminum oxide, and also to determine how they may be specifically obtained by methods capable of reproduction. Thus it has been found that the alumina preparate *C* obtained by cautious precipitation with dilute ammonia, corresponds to

[1] B. **56**, 149, 1117 (1923); **57**, 58, 1082 (1924); **58**, 2448, 2458 (1925).
[2] B. **56**, 149 (1923).
[3] B. **58**, 2448, 2458 (1925).

the composition of an ortho-aluminum hydroxide, having the formula $Al(OH)_3$. This alumina gel (C_α) which is obtained under the mildest conditions, is characterized, however, by the ease with which it suffers alteration; in aqueous suspension it is changed after a short time into a second compound (C_β), which while likewise unstable, is more stable than the α modification and is then gradually transformed into the third modification (C_γ). With these transformations, which are to be attributed to a chemical alteration of the molecule, whether it is an isomeric or a polymorphic phenomenon, there is connected an alteration of the basic and acid properties and of the adsorption behavior of the gels; they are not to be explained by alterations in the colloid state. Thus the alumina varieties *A* and *B* reveal themselves as poly-aluminum hydroxides. It was further found possible to obtain from the ortho-hydroxide of aluminum, as likewise by the conditions of direct precipitation with long continued action of ammonia and a higher experimental temperature, a further definite hydrate, whose composition corresponds to the formula of a meta-hydroxide, AlO.OH; this hydrate, which has proved to be specially effective for the separation of the saccharase and maltase in yeast autolysates, possesses neither acid nor basic properties.

Alternate Use of Various Adsorption Agents.—A limit is placed on the further purification of any enzyme by the use of an adsorption medium, after separating out the other enzymes associated with it, by the fact that the absorbent takes up at the same time as the enzyme a larger quantity of non-enzymic substances, whose affinity, like that of the enzyme, may depend upon an opposite electrical charge. In such cases success has been obtained by the change in polarity of the adsorption medium, the exchange of an electropositive for an electronegative adsorbent. Thus preparations of yeast saccharase, previously purified by alumina adsorption and still containing about three-fourths of their amount of yeast gum, may be completely freed from this carbohydrate by subsequent adsorption on kaolin. The enzymic concentration is increased thereby about five-fold—it becomes about 500 times as great as in the yeast itself. In pancreatic lipase also the successive use of an electropositive and an electronegative adsorbent permits much greater increase in the purity than does the repeated use of one of the adsorbents alone. Still further progress has been made in the case of the pancreas lipase by the use of indifferent

organic adsorption media, such as cholesterin or tristearin, which in this case appear to have a more strictly selective action than kaolin or aluminum hydroxide. These phenomena must be ascribed to a chemical adsorption related to the specific adsorption, a concept which appears to be supported by the fact that the affinity of the specifically active group of the lipase in these adsorbates is brought into extremely intimate association with the adsorbate, so that its activity is appreciably weakened, although the enzyme can be freed from them again in an active form.

Limitations of the Adsorption Procedures.—The increase of the enzymic degree of purity, obtainable by preparative methods, should be determined in each case by the absolute concentration of the enzyme in the original material, which it is not yet possible to determine. If in the best yeast saccharase preparate an increase in purity of over 3,000 times is obtained, but with animal lipase the degree of purity is raised only 250 times, no basis can be taken from this comparison for the absolute enzymic concentration of both preparates. The goal of preparative enzyme chemistry is to carry the purification to such a point that no increase in its degree can be realized by further operations. This goal has in no case been obtained but the information gained in the more recent preparative researches upon yeast saccharase justify the view that the success attainable by the adsorption methods, as hitherto worked out, is in a certain sense only a limited one, and that such success from a certain degree of enzymic purity on, appears to be compensated by the increasing lack of stability in the enzyme itself.

Purification of Insoluble Enzymes.—The applicability of purification processes so far described, including fractional precipitation or adsorption and elution, are limited exclusively to soluble enzymes. The purification of insoluble enzymes, for which the fat-cleaving enzyme of plant seeds may be taken as a characteristic example, requires a different preparative method and this method must be confined to increasing the enzymic concentration by mechanical enrichment or to a dissolving out of the associated substances. For this purpose, there are only a few methods that are sufficiently mild to avoid a destruction of the enzymic activity.

In order to prepare ricinus lipase, which will serve here as an example, Willstätter and Waldschmidt-Leitz,[1] combine several

[1] H. **134**, 161 (1923–24).

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

special conditions which are based upon a strikingly marked dependence of the enzymic activity upon the colloidal state. The fat-free form of the enzyme is found to be very unstable with respect to the action of water or glycerine, though it suffers no loss of activity in the presence of oil. In oil and water emulsions, on the other hand, it displays an excessive sensibility towards transformation into the dry and fat-free form. To render the enzymic substance susceptible to the action of aqueous reagents, while retaining its lipatic activity, only one method is applicable, one which leaves the lipase in contact with oil throughout all purification processes. Such a method was first followed in principle by Hoyer; [1] it is based on the separation of the enzyme from the aqueous emulsion of the oil-bearing seed in the form of a fat and water containing cream, such as is obtained by centrifuging. Enzyme preparations of increased purity have been obtained from such "enzyme creams" under the action of dilute aqueous reagents, such as alkaline, acid, or salt solutions. It is true that certain difficulties are encountered in obtaining lipatically active dry preparations from these creams, whose separation after treatment with the chosen reagent is accomplished by centrifuging. Such difficulties arise from the sensibility of the enzyme with respect to drying operations but are overcome by the use of specially mild processes of drying, either with the help of dispersoids of great specific surface, or by a very gradual removal of the water and oil content through gentle drying in a moderately warm air current or by the slow dilution of the residuary oil-containing enzyme suspension in an atmosphere of ether. By this means, an increase of the lipatic concentration to an amount more than fifty times that of the seeds was obtained in the most successful examples.

This purification, which must be regarded as very imperfect, and which has not permitted a complete separation of protein substances, is to be valued only because it may furnish an example for the preparative method as applied to insoluble enzymes. For the purpose of producing pure enzymic individuals and for their chemical characterization, we must for the present confine ourselves to water-soluble substances and be content with refining and extending the preparative methods therein employed.

[1] H. **50**, 414 and particularly p. 430 (1906–07).

SPECIAL SECTION

In the course of the last few years many special researches have appeared dealing with the action and behavior as well as with the occurrence of enzymic substances. It would exceed the limits and the purpose of this volume to attempt a complete tabulation and description of all the separate works which deal with enzyme research. This section, will be devoted to the special treatment of representatives of the various groups of enzymic substances and will take up the description of only the most important and best-studied enzymic catalyzers.

I. ESTERASES

The general action of esterases is characterized by the cleavage or synthesis of carboxylic acid esters according to the equation

$$R \cdot COO \cdot R + H_2O = R \cdot COOH + HO \cdot R,$$

and, in this regard by the transformation of both the esters of simple alcohols and polyvalent alcohols, e.g., esters of the fatty acid glycerides. As proposed by W. Connstein,[1] the esterases are most conveniently classified according to their origin, into animal and plant esterases, although as yet we do not have sufficient basis to make a general distinction between the esterases that are most effective in converting the esters of the lower fatty acids and the lipases that are most effective in converting the glycerine esters of the higher fatty acids.

1. ANIMAL ESTERASES

a. Pancreas Lipase

Pancreas lipase is secreted into the intestine by the pancreas along with other important enzymes involved in animal digestion,

[1] Ergebn. d. Physiol. **3**, 194 (1904).

in order to effect here the preliminary saponification of the fats taken in with the food, previous to absorption. This enzyme must be considered as a true lipase, specific for the transformation of glycerine esters, whereas its action upon the esters of simpler alcohols is retarded.

Methods of Determination.—Numerous methods for demonstrating the presence of lipase and for measuring its activity have been published. These are based in part on the volumetric determination of the fatty acids formed by cleavage, and in part on the variation of the surface tension which occurs during the saponification of the glycerine esters of the lower fatty acids. In the older literature, the glyceride converting power of the enzyme was measured merely under the adventitious conditions in which the enzyme occurred. Willstatter, Waldschmidt-Leitz and Memmen [1] have now recognized and taken into consideration the necessity of placing the lipase in a suitable system, in which compensators act to equalize the differences in activity occasioned by the nature and the quantity of casually occurring substances. It has been found that the action of the pancreas lipase, towards true fats, is influenced to a spectacular degree by the concomitant substances as well as by the reaction products, whereas the importance of H-ion concentration to the activity of the enzyme is less pronounced. The effect of the concomitant and addition substances and of the reaction products varies with the reaction; thus, calcium salts, gall salts or proteins are activating in an alkaline medium, but indifferent or even inhibiting in an acid medium. The activations observed are to be ascribed to an improvement in the adsorption relations, the inhibitions to disturbances in these relations. The establishment of a contact between the water-soluble enzyme and the insoluble substrate is necessary for its action. This is expressed by the formation of complex adsorbates when activating in an alkaline medium, and, correspondingly, by a destruction of its association with oil, when the inhibition phenomena are observed in acid reactions. Hence to obtain a method by which we may follow the quantity of the enzyme and its yield from the gland to the pure preparation we must exclude chance occurrences in the lipatic system, in so far as they exert an effect upon the degree of the action; this object is attained by the process of compensating activation or

[1] H. **125**, 93 (1922–23).

inhibition. Such an idea has culminated in the development of four special procedures for the determination of lipatic enzymes, all of which have been found reliable as means for following the enzyme content during preparative purifications. These procedures are:

1. The alkalimetric determination of lipatic action in a system of activators with varying *p*H, i.e., the cleavage of olive oil with activation by calcium chloride and albumin, the initial *p*H being 8.9.

2. Alkalimetric determination in alkaline medium with a constant *p*H of 8.9, with olive oil serving as the substrate and calcium chloride and albumin as the compensating activation.

3. Alkalimetric determination with inhibition in acid medium, i.e., oil-cleavage at a constant acid reaction (*p*H − 4.7) and inhibition by albumin.

4. Stalagmometric determination of tributyrin hydrolysis according to the method of Rona and Michaelis,[1] based on the measurement of the variation in surface tension at *p*H = 8.6 and with activation by calcium oleate and albumin.[2]

Of these processes, only the second, the conduct of which requires the use of high buffer concentrations, has the disadvantage that in testing pure enzyme preparations a destruction of the enzyme is perceptible even during the period of determination, and that this necessarily affects the accuracy of the determination. Since no precision is to be expected under these experimental conditions with respect to the time curve of hydrolysis and the proportionality between the reaction velocity and the enzyme quantity, the measures taken for the quantity and concentration of the enzyme depend upon the relation between the enzyme quantities and the degrees of cleavage which are attained during given periods of time.

The lipase unit is the quantity of lipase that will transfrom 24 per cent of 2.5 g. olive oil (saponification number = 185.5) in one hour under the conditions of the first method of determination; these conditions are: a volume of 13 ccm. containing 2 ccm. n NH_3—NH_4Cl—buffer of *p*H = 8.9, and as activators 10 mg.

[1] Bio. Z. **31**, 345 (1911); P. Rona, ibid. **32**, 482 (1911); H. Davidsohn, ibid. **45**, 284 (1912); **49**, 249 (1913).

[2] R. Willstätter and Fr. Memmen, H. **129**, 1 (1923).

$CaCl_2$ and 15 mg. albumin. This amount of enzyme is contained in about 1 cg. of a good sample of dried pancreas gland. The diagram (Fig. 7) is taken from the investigation by Willstätter, Waldschmidt-Leitz and Memmen and expresses the relation between lipase quantity and degree of saponification. The curve was obtained with the following quantities of a good pancreas sample carefully dried:

Gland, mg.	Per cent conversion in one hour
1.33	7.4
3.99	13.1
7.98	20.1
10.00	24.0
13.33	25.7

This explains how the number of enzyme units in a given amount of sample may be determined from an alkalimetrically determined degree of saponification (so long as the degree of saponification does not exceed 24 per cent, preferably it should be between 10 and 24 per cent).

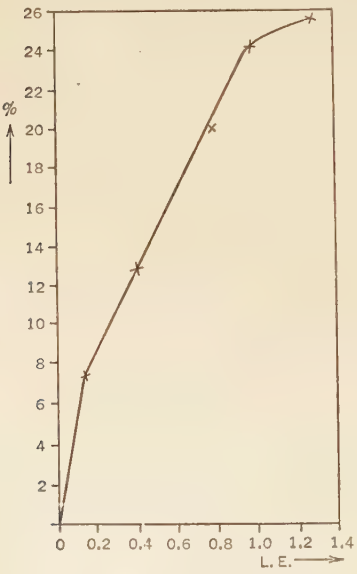


Fig. 7.—Lipase Quantity and Degree of Saponification.

The "lipase value (L. W.)" serves as a measure for the degree of purity of the lipase; it is given by the number of lipase units in 1 cg. of the preparate. For example:

6.25 mg. of pancreas sample gave 15.1 per cent hydrolysis, which, according to Fig. 7, corresponds to the action of 0.52 lipase units. The lipase value of this gland material is

$$\frac{0.52 \cdot 10}{6.25} = 0.83$$

Another scale for lipase quantity, independent of the one given above, is based upon the stalagmometric observation of tributyrin-hydrolysis according to the fourth method of determination.

This scale according to Willstätter and Memmen is based on the "butyrin-unit (B.E.)," which corresponds to that quantity of enzyme which occasions in 50 min. a decrease of the drop number by 20 at 20° C. under the given conditions. This drop number is about half the difference between the drop number of pure tributyrin solution and pure water. The given conditions are: a volume of 60 ccm., containing 56 ccm. of saturated tributyrin solution, 2 ccm. n NH_3 — NH_4Cl — buffer of pH = 8.6, and as activators 10 mg. $CaCl_2$, 10 mg. sodium oleate and 30 mg. albumin.

Fig. 8 illustrates the time course of tributyrin hydrolysis with the enzyme quantity designated as 1 B.E. With an unknown sample, which should be so diluted as to have approximately the same activity, the B.E. is equal to $50/t$, t being the time required for this sample to cause a decrease of 20 in the drop number.

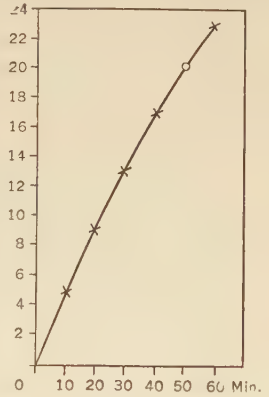


Fig. 8.—Time Course of Tributyrin Cleavage by the Enzyme Quantity which is Designated as 1 B.E.

The relation of the unit for tributyrin cleavage to the unit for oil cleavage, which in a tested and compared sample of dried swine pancreas corresponded to the ratio 1 B.E. = 0.001 L.E., cannot, however, be taken as a general formula, since it is dependent upon the origin of the materials: hence, for the measurement of lipase quantities from different sources a given method of determination must be employed.

Preparative Purification.—The preparative purification of pancreas lipase has been carried out by Willstätter and Waldschmidt-Leitz,[1] in a series of examples. The dried and fat-extracted pancreas gland of swine, the enzyme content of which is highest, was used as starting material. A good yield of lipase could be carried over into the solution by treatment with water or glycerine, whereas only a small portion of the enzyme can be extracted by these methods from the fresh gland substance. The mechanically fragmented gland tissue was dried by agitation with acetone and ether, with no appreciable injury to the enzyme; it was proved on the other hand that the older process first used

[1] H. **125**, 132 (1922–23).

by Pottevin [1] of drying by alcohol and ether is accompanied by very considerable loss of activity.

For extracting the enzyme from the dried and pulverized gland substance, the use of glycerine was found to be much better than aqueous extraction. The lower degree of purity and of stability of the enzyme render aqueous extracts unfit for preparative purposes but the lipase is stabilized by the presence of glycerine. It is therefore requisite for the conduct of preparative purification to maintain a certain degree of glycerine concentration in order to avoid enzymic losses. The lipase is clearly dissolved in the glycerine extracts of the gland, which are separated from insolubles by centrifuging or filtration. The reports of the earlier researchers [2] to the effect that the enzyme was merely suspended in glycerine solutions and not dissolved and that it could be precipitated therefrom by dilution with water have proved to be erroneous; those views must be ascribed to adsorption of the enzyme in suspended fats in very impure extracts.

Adsorption Behavior.—In both the aqueous and the glycerine extracts of the gland, the lipase is accompanied by the entire quantity of the starch-splitting enzyme and by the greatest part of the tryptic enzyme, from which it cannot be separated by means of such precipitants as alcohol or acetone, the effect of these being a destruction of the lipatic activity. Lipase was first successfully obtained in enzymically integral form by the use of adsorption methods based upon the specific adsorption affinities of the three enzymes. The adsorption behavior of lipase is highly non-specific, the enzyme being easily taken up by either acid or basic adsorbents; it may even attach itself to amphoteric substances, e.g., to proteins, and may be carried over in this manner, as is evident from its increased activity, into an adsorption system favorable to oil-cleavage. Furthermore, the adsorption of the enzyme is found to be but slightly or not at all dependent on the presence of associated materials; thus in the crude as well as in the purer solutions no appreciable difference is shown in its qualitative or quantitative adsorption behavior towards alumina.

However, the acid properties of lipase are more strongly marked than those of amylase and trypsin; it may be successfully

[1] Bl. **35**, 693 (1906).

[2] See O. Rosenheim, J. Physiol. **40**, XIV (1910); C. A. Pekelharing, H. **81**, 355 (1912); P. Rona and Z. Bien, Bio. Z. **64**, 13 (1914).

isolated from the enzyme mixture by adsorption on the basic adsorbent alumina, preferably in acid reaction. It is true that under the influence of certain concomitant substances small portions of the accompanying enzymes which were carried over were found again in the elutions obtained from alumina adsorbates with dilute alkali or with alkaline phosphate; but a repetition of the adsorption process in the acidified elution sufficed to remove their final residues. The degree of purity of the lipase, however, was not appreciably increased in the new elutions, its lipase value was increased only about 30-fold. But adsorption with an electronegative adsorbent, kaolin, from acid solution, led to a further very considerable increase in the enzymic concentration: in the alkaline elutions to more than 200-fold that of the source materials. Still more sharply selective is the action of indifferent organic absorbents such as tristearin or cholesterin; after they have been dissolved out, as with benzol, the enzyme is obtained in the highest degree of concentration thus far measured, corresponding to the lipase value 240. In Table XXIV, taken from the communication by Willstätter and Waldschmidt-Leitz,[1] is given a summary of the varying degrees of purity obtained in the different stages of the preparative isolation.

TABLE XXIV

PHASES OF THE ISOLATION AND DEGREE OF PURITY OF PANCREAS LIPASE

(Dried Gland: *L. W.* = 0.81)

Preparate	*L. W.* Determined by Dialysis	*L. W.* Determined by Isolation with Stearine
Glycerine extract, clarified	3.2	
Adsorbed once with alumina	9.0	72
Adsorbed twice with alumina	23.1	
Adsorbed once with alumina and kaolin	25.8	119
Adsorbed twice with alumina and once with kaolin	207.0	240

The purification of pancreas lipase has not been carried to the point where its lipase value is constant. Nevertheless, it

[1] H. **125**, 132 and particularly p. 192 (1922–23).

has been shown that in the purer preparates there can no longer be detected any intermixtures of members of known groups of natural organic substances, of proteins or carbohydrates. Characteristic chemical reactions of the enzymic substances were observed; the nitrogen content, which underwent a loss in the course of purification, amounted in the purest preparate to 10.5 per cent, whereas the observed phosphorus content of 0.05 per cent must be regarded as being circumstantial and of practically no significance.

b. Stomach Lipase

Hydrogen-ion Dependence.—Observations regarding the presence of a fat-splitting enzyme in the stomach are very old. However, the question as to its special existence as distinct from the pancreatic enzyme was first studied by Volhard [1] and his students. At the same time a positive distinction between the gastric and the pancreatic lipases appears to have been first made possible by the researches of Davidsohn; [2] these resulted in the finding that the enzyme of the human stomach, contrary to that of the pancreas, had a much stronger action in an acid reaction than in an alkaline reaction, and possesses a moderately broad zone of optimum action, between *p*H 4 and 5. But the distinction between the two enzymes from this point of view has not been confirmed. From the researches of Willstätter [3] and his colleagues with respect to the stomach lipase of various animals and its comparison with the pancreas lipase, it has been found that the *p*H dependence of the stomach enzyme varies with the animal under observation, and that it is distorted and falsified by the concomitant substances which are capable of inhibiting or activating according to the reaction. In the course of preparative purification the observed differences disappear with the separation of these concomitant substances. The gastric enzyme gradually approximates the pancreas lipase in its behavior towards activa-

[1] Z. f. klin. Med. **42**, 414; **43**, 397 (1901); W. Stade, Hofm. Beitr. **3**, 291 (1902); A. Zinsser, ibid. **7**, 31 (1905); A. Fromme, ibid. **7**, 57 (1905).

[2] Bio. Z. **45**, 284 (1912); **49**, 249 (1913).

[3] R. Willstätter and Fr. Memmen, H. **133**, 247 (1923–24); R.Willstätter, F. Haurowitz and Fr. Memmen, H. **140**, 203 (1924); F. Haurowitz and W. Petrou, H. **144**, 68 (1925).

tors as well as in its dependence on the *p*H; the zone of optimal action changes, for example, with the lipase of the dog's stomach, from the acid into the alkaline region. The assumption of a difference between the stomach and pancreatic lipase among the different animals investigated finds no support on the basis of their *p*H dependence. It is only the different stereochemical specificity of the two enzymes which seems to be responsible for their difference as will be explained later.

Measurement.—We do not as yet possess the same sure methods for measuring the stomach lipase and comparing its quantities in varying degrees of purity as we do in the case of the pancreatic enzyme. The enzyme occurs in a complex of varying compositions made up of activating and inhibiting substances, whose action, as well as that of the activators to be used, is manifested differently with varying H-ion concentrations; no success has been attained thus far in compensating their influences. Hence, the scale employed by Willstätter, Haurowitz and Memmen,[1] which was derived from the stalagmometric measurement of the hydrolysis of tributyrin, corresponds not to actual but merely to apparent enzyme quantities, which are expressed as B(e) (that is, apparent enzyme units).

Increase in Degree of Purity.—The mucous membrane of the dog stomach served for the preparation of the enzyme, since it is present here in large amounts as compared to the other parts of the stomach; its extraction is effected by dilute ammonia. In order to carry out its further purification it was precipitated by dilute acetic acid; also the precipitate was dissolved in alkali and subjected to electro-dialysis, after which the enzyme was again found in the precipitate formed; finally the solution was subjected to another pre-adsorption with kaolin, this being done in a neutral reaction, after which there was seen still another characteristic increase of purity on the part of the enzyme remaining in the mother liquor. The latter increase was expressed not so much in the analytically determinable enzymic concentration as rather in the separation of characteristic, intermixed substances which distorted or disguised the true enzymic properties.

In Table XXV the yields obtained by Willstätter, Haurowitz and Memmen, in the preparative purification of the enzyme, are compared with the increase in the degree of purity. The apparent

[1] H. **140**, 203 and particularly p. 207 (1923–24).

enzyme increases observed in the measurement in an alkaline zone as well as the decrease of the enzyme quantities in acid reaction, are to be explained by the separating out of alkaline inhibiting and acid activating substances, which may also be recognized in the alteration of the *p*H dependence of the enzyme.

TABLE XXV

Degree of Purity and Yield of Lipase from the Dog's Stomach

Preparate	Degree of Purity B.(w). at *p*H = 6.3	Yield, Per Cent	Degree of Purity B.(w). at *p*H = 8.6	Yield, Per Cent	Optimum Activity, *p*H	Minimum Activity, *p*H
Mucous membrane extract.....	3.9	(100)	0.4	(100)	5.5–6.3	8.6
After precipitation by acetic acid....	10.9	95	2.4	200	5.5–6.3	8.6
After electrodialysis.	35.1	31	25.8	220	6.3–7.1	4.7
After preadsorption. with kaolin.....	37.0	24	27.0	232	7.1–7.9	4.7

Similar alterations of the *p*H-dependence have likewise been observed by Haurowitz and Petrou [1] in purifying human stomach lipase.

c. Liver Esterase

Kinetics.—Of the esterases of the animal tissue that of the liver has been the most studied. Its pronounced affinity for the esters of simple alcohols has especially occasioned kinetic measurements. While the earlier researches by Kastle and Loevenhart [2] on the reaction course of enzymic, ethyl butyrate and ethyl acetate hydrolysis yielded no simple obedience to law, on the other hand, Knafft-Lenz [3] found that the kinetics of ester hydrol-

[1] H. **144**, (1925).

[2] Am. **24**, 491 (1900); see also J. H. Kastle, M. E. Johnston and E. Elvove, Am. **31**, 521 (1904) and R. O. Herzog in C. Oppenheimer, Die Fermente und ihre Wirkungen, 4. Aufl. p. 1021. (1913).

[3] Medd. K. Vetenskapsakad. Nobelinst. **6**, 1-18, Nr. 2 (1922); Arch. exp. Pathol. u. Pharmakol. **97**, 242 (1923).

ysis with a constant acidity corresponded to a reaction of zero order of magnitude, or that there is a direct proportionality between the transformation and the time. The deviations from this relation as reported in the earlier works are ascribed not only to the inconstancy of the *p*H but also to the strongly inhibiting action of the butyric acid formed. The dissociation constants of the esterase-ester compound are very small, according to Peirce,[1] and the affinity of the enzyme to its substrate is therefore very great; correspondingly, the capacity of the enzyme is found to be relatively independent of the concentration of the substrate.

Comparison with Pancreas Lipase.—On the other hand, Willstätter and Memmen [2] have pointed out that in the case of pancreatic lipase a much smaller affinity to simple esters has been observed and that here, even in the saturated solution of the ester, not all of the enzyme appears to be bound to the substrate. This difference in the dependence upon the substrate of the two enzymes corresponds to a difference in the reaction process, which exhibits no proportionality for the pancreas lipase between the time and the transformation but corresponds, rather, with an approaching proportionality between absolute cleavage and substrate concentration, to an equation of the first order. The optimal zone of action of the enzyme, according to Knaffl-Lenz, lies between *p*H 7.8 and 8.8, therefore in a weakly alkaline reaction.

Purification.—The preparative purification of the liver enzyme in the earlier works has not been carried very far. Attempts have been confined principally to the dialysis of extracts of raw liver pulp or to the subjection of these extracts to fractional salting out procedures. The preparates obtained have been inadequately characterized with respect to their enzymic yield and concentration. In the more recent researches by Willstätter and Memmen, purification has been attempted by means of adsorption with alumina and with kaolin, and subsequent electrodialysis, the degree of purification being measured by the stalagmometric observance of tributyrin hydrolysis. In the best examples the preparates reached an enzyme concentration of about 50 times that of the liver powder. At this stage the concomitants were predominately proteins and their degradation products.

In further extending these experiments Kraut and Ruben-

[1] Am. Soc. **32**, 1517 (1910).

[2] H. **138**, 216 and particularly p. 234 (1924).

bauer [1] have found that, if these associated materials can be removed before adsorption, the method is capable of yielding a very high degree of purity. This desired fractionation can be accomplished by dialysis. Then by the alternate use of kaolin and alumina the enzyme can be completely freed from protein. Such solutions, apparently, are not to be classified among any of the known organic groups. The degree of purity in the best preparates is 128 times that of the dried organs.

Upon attaining these higher degrees of purity, a striking fact is to be observed; namely, the enzyme suffers an abrupt loss in stability as soon as the solution fails to exhibit the Millon reaction. At this stage of purification, a loss of 50 per cent can occur overnight, although the enzyme had previously been characterized by a much higher degree of stability. This transition from fair stability to high instability is more abrupt than with any enzyme so far studied.

From these results it has been assumed that the stabilizing agent of liver esterase is actually the substance which gives the Millon reaction and is not one of the other concomitants removed during the purification process. Certain further evidence indicates that a tyrosine-containing protein degradation product is the effective agent. Such an assumption is supported by other observations with different enzymes. Willstätter and Schneider note that their most stable saccharase preparate gives a particularly strong Millon test and Sherman has demonstrated the protective action of various amino acids on purified pancreas amylase. Numerous similar instances have been reported.

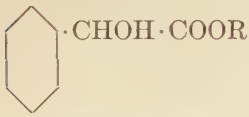
Induction Period.—In experiments on the hydrolysis of mandelic acid ethyl ester by liver esterase, a certain induction period has been noted before the commencement of the normal saponification process. For example, only a small degree of saponification will take place during a period of about 60 minutes, after which time the course of ester hydrolysis sets in with a rapid velocity.

Such induction phenomena have already been observed in a number of ferment reactions. Tammann [2] caused a solution of emulsin and amygdalin, which had reached the end state, to act on salicin. Upon mixing both solutions the hydrolysis of the glucoside began only after a period of 10 minutes. Induction

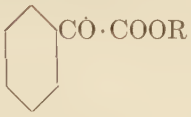
[1] H. **173**, 103 (1928). [2] Z. Physikal. Chem. **3**, 25 (1889).

periods of the same order of magnitude have been noted by Abderhalden and Fodor [1] in a splitting of peptides by yeast juice. The cause of these particular phenomena is unknown. A better understanding has been reached as to the cause of induction in the alcoholic fermentation of sugar by dry yeast and by yeast maceration juice, which can take 24 hours and longer. According to Meyerhof,[2] in the case of maceration juice fermentation immediately sets in upon addition of small quantities of hexose phosphate (0.0002 moles). With dry yeast, according to investigations of Abderhalden and Fodor [3] and of Sobotka,[4] this phenomenon is probably connected with the revival of the yeast cells.

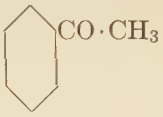
In the case here of mandelic acid ester the exclusive cause of the reaction retardation has been traced to the presence of an impurity in the substrate.[5] This impurity, benzoyl formic acid ethyl ester, has practically the same boiling point as the mandelic ethyl ester and attempts at their separation by fractional distillation were unsuccessful. Upon recrystallization from petroleum ether,



Mandelic acid ester



Benzoyl formic acid ester
Phenyl glyoxalic acid ester



Acetophenone
Phenyl methyl ketone

however, the impurity was removed and the latent time period disappeared completely. Synthetic mixtures of the substrate and the impurity, the keto acid ester, exhibited latent times which were directly proportional to the amount of the impurity.

Affinity to Keto-carboxylic Acid Esters.—The cause of the induction is explained by the very marked affinity of liver esterase to phenyl glyoxalic acid ester and the very much lower decomposition velocity of the products formed. In spite of the great excess of mandelic acid ester, the total quantity of the enzyme is bound by the keto acid ester and is made available for the hydrolysis of the mandelic acid ester only with extreme slowness, namely, in that degree to which the keto acid ester is decomposed.

[1] Fermentf. **1**, 533 and particularly p. 578 (1916).
[2] H. **102**, 1, 185 (1918).
[3] Fermentf. **5**, 138 (1921–22).
[4] H. **134**, 1 (1923–24).
[5] R. Willstätter, R. Kuhn, O. Lind and F. Memmen, H. **167**, 303 (1927).

Other keto acid esters, such as oxal-acetic ester and benzoyl acetic ester, give rise to the induction phenomenon. Since neither the ester group alone (benzoic acid ethyl ester, phenyl acetic acid ethyl ester) nor the keto group alone (acetophenone) occasion the induction phenomenon, the observations are ascribed to the constitutive individuality of the keto carboxylic acid esters and to their relations to liver esterase.

THE SPECIFICITY OF ANIMAL ESTERASES

Relative Specificity.—The discovery made by Loevenhart,[1] according to which the ester-splitting enzymes of the pancreas and liver must be regarded as different enzymic individuals because of their quantitatively different specificity for substrates of different constitution, has found a valuable extension in the researches of Willstätter and Memmen.[2] It has been found that pancreas lipase, independent of the enzymic degree of purity, is a very active enzyme for the cleavage of higher glycerides, e.g., the natural fats, whereas the liver enzyme, which has been found unadapted to this purpose, is especially effective for the hydrolysis of esters of univalent alcohols such as butyric acid ester. "The pancreas enzyme may be designated as a *lipase*, which is also capable of hydrolysing simple esters, and the liver enzyme as an esterase, which is only capable of converting fat in a very small degree." These differences of specificity are very great and cannot be easily explained if the identity of the two enzymes is assumed. For instance, in order to accomplish the same hydrolysis with a sample of dried swine liver as with 1 cg. of dried pancreas, 106 g. of the liver were required for oil cleavage, whereas, for the cleavage of tributyrin only 1 g. was required and for that of methyl butyrate only 4 mg. Thus it is shown that the stomach lipase resembles the pancreas lipase, not only in its physiological function, but also in its constitutional specificity, even though its occurrence is much more limited; "it is a true lipase." [3]

Stereochemical Specificity.—Stereochemical specificity, which must be regarded as a special case of general structural specificity,

[1] J. Biol. Chem. **2**, 427 (1906–07).

[2] H. **138**, 216 (1924).

[3] R. Willstätter, F. Haurowitz and Fr. Memmen, H. **140**, 203 and particularly p. 206 (1924).

was first observed for an ester-splitting enzyme, liver esterase, by Dakin [1] in racemic esters of the mandelic acid group. Warburg [2] as well as Abderhalden [3] have sought to utilize this phenomenon for preparative purposes in the case of racemic amino acid esters. The configurative specific structure is less strict in the esterase in general than it is, for example, in the glucoside splitting enzymes, i.e., both antipodes are commonly hydrolyzed, but with different velocity. In comparing the stereochemical specificity of the three most important animal esterases, from pancreas, liver and stomach, Willstätter and his colleagues have found that none of the three enzymes is identical with another as to configurative individuality. Although, it is true, cases are observed in which two of the enzymes examined, e.g., pancreas lipase and liver esterase, prefer components of identical activity, in many other cases, on the contrary, the components of opposite rotation are saponified more rapidly by the two enzymes.

Table XXVI shows, by characteristic examples, the differing steric capacities of the three esterases together with that of the ester splitting enzyme from *aspergillus oryzae*; [4] the signs refer to the direction of rotation of those components which are rapidly saponified.

TABLE XXVI

Optical Specificity of Animal Esterases

Racemic Substrate	Enzyme from			
	Pancreas	Stomach	Liver	*Aspergillus Oryzae*
Mandelic acid-ethyl ester....	−	+	+	+
Phenyl chloracetic acid-methyl ester.....	−	+	−	−
Phenyl-methoxy acetic acid-methyl ester.....	−	.....	+	+
Tropic acid−methyl ester...	+	+	−	−

[1] J. Physiol. **30**, 253 (1903–04); **32**, 199 (1905).

[2] B. **38**, 187 (1905); H. **48**, 205 (1906).

[3] E. Abderhalden, H. Sickel and H. Ueda, Fermentforschung **7**, 91 (1923).

[4] See R. Willstätter and H. Kumagawa, H. **146**, 151 (1925).

Evidence has recently been obtained indicating, further, that this stereochemical specificity is truly a property of the enzyme itself and not a function of the concomitant activating or inhibiting substances. Extensive purification experiments with the above 3 animal enzymes does not cause any change in the sign of the stereochemical specificity.[1] In that these differences are maintained in the highly purified preparates, it may be well assumed that these esterases are inherently different.

Also in the case of tartaric acids, Dawson, Platt and Cohen [2] find that the action of liver lipase and pancreatic lipase are different. Unlike pancreatic lipase, liver lipase hydrolyses the esters of d-tartaric acid more readily than those of the l-acid.

Toxic Susceptibility.—For determining the identity or difference of ester splitting enzymes, Rona [3] and his co-workers have also turned to the specific influences exerted by certain alkaloids and other poisons. Their conclusions are based on the assumption that the toxic susceptibility of the enzymes is not conditioned by their circumstantially coexistent substances, i.e., their reaction system, but by their individual chemical and physical constitutions. That is, this sensitivity remained even after purification, although not a very extensive one. Meanwhile, Willstätter and Memmen [4] have pointed out that adventitious substances can have a marked influence on the action of the poisons. A solution of pancreas lipase, which was insensitive to quinine, was strongly inhibited by this alkaloid after addition of egg albumin; moreover, the toxic action of the alkaloid varied from preparate to preparate. These poisoning phenomena can no longer be regarded as adequately characteristic for a distinction between different enzymic individuals.

2. PLANT ESTERASES

a. Ricinus Lipase

The lipase of the oleaginous seeds of *ricinus communis*, a typical example of the fat-splitting enzymes of the plant kingdom, was

[1] R. Willstätter, E. Bamann, and J. Waldschmidt-Graser, H. **173**, 155 (1928); R. Willstätter, R. Kuhn and E. Bamann, B. **61**, 886 (1928).

[2] Biochem. J. **20**, 533 (1926).

[3] Bio. Z. **111**, 166 (1920); **118**, 213; **130**, 225 (1921); **134**, 108, 118 (1922); **146**, 28 (1924).

[4] H. **138**, 216 and particularly p. 241 (1924).

first described by Sigmund [1] and Green.[2] As a true intracellular enzyme this lipase is specially distinguished by its complete insolubility in aqueous media, and it differs therein from most of the other ester-splitting enzymes of the plant and animal world.

Working Conditions.—According to Armstrong,[3] ricinus lipase is strongly active only in the case of true fats, whereas, it splits the lower esters with relative sluggishness; hence it is a pronounced lipase. The methods used for its measurement, accordingly, are based predominantly upon the saponification of true fats. The lipase of the resting, ungerminated plant seed, which, as a rule, has served for experimentation, possesses an optimum action in acid reaction, i.e., according to Haley and Lyman,[4] as also according to Willstätter and Waldschmidt-Leitz,[5] at the H-number of 4.7 to 5.0. No simple principles are to be expected for their action against true fats, as regarding the reaction course with respect to the relations between enzyme quantity and transformation; and neither have such principles been observed. Furthermore, its performance in accordance with the peculiar physiological conditions of the reaction of the enzyme is not affected by activators. The scale instituted by Willstätter and Waldschmidt-Leitz, for measuring the enzyme and for the control of its preparative isolation depends therefore on the empirically derived relations between the quantities of the enzyme and the degree of saponification, which is observed in a series of oils after a definite lapse of time. The latter is established by alkalimetric determination of the fatty acids formed. These are given in Fig. 9.

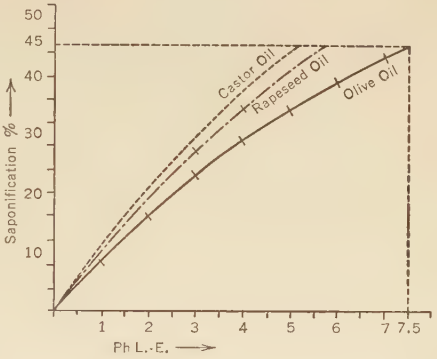


FIG. 9.—Lipase quantity and degree of saponification.

[1] Monatshefte f. Chemie **11**, 272 (1890).
[2] Proc. Roy. Soc. B. **48**, 370 (1890).
[3] Proc. Roy. Soc. B. **76**, 606 (1905); **78**, 376 (1906).
[4] Am. Soc. **43**, 2664 (1921).
[5] H. **134**, 161 (1923–24).

The "Phytolipase Unit (Ph.L.E.)" has been chosen as a measure for the ricinus lipase. It is that quantity of enzyme which, under the conditions of the determination, splits 7.5 per cent of the substrate in 20 minutes. The conditions are a *p*H of 4.7, a temperature of 20° and 2.5 g. of substrate (olive oil, saponification value = 185.5). The "Phytolipase Value (Ph.L.W.)" designates then the enzymic concentration or the number of Ph.L.E. in 1 cg. of the preparate.

Procedure for Purification.—The preparative purification of the enzyme, of which there are several examples in the literature, is rendered exceedingly difficult by its insolubility and by its great sensibility with respect to alterations of the colloidal state. This sensitivity must be attributed to its linkage with an insoluble colloidal carrier, belonging probably to the protein group. The lipase not only has proved to be entirely insoluble in aqueous or organic media, but its activity is almost entirely destroyed in the dried, fat-free form under the influence of aqueous solvents, and also of many organic solvents, as glycerine or glycol. On the other hand, the enzyme possesses in oil and water emulsions an extremely great sensibility in undergoing transition into the dry and fat-free form. Hence there are but very few processes which are sufficiently mild to lead to an enrichment of the enzymic substance from the seed material. To these belong, for example, the methods recommended by Nicloux[1] and by Hoyer.[2]

These methods consist in fractionating, according to the specific gravity, the oleaginous seeds which have been made into a paste with oil or oil solvents. The specific gravity of the oil or oil solvents is so adjusted that the enzyme-rich portions of the seed may be centrifuged out. However, in the fat extraction, by means of organic solvents, of those resulting fractions which are richest in enzyme, as well as in the oil extracting of the seed mass itself, there are appreciable losses in the enzymic action and the further purification of the enzyme from these stages onward is prevented by its sensitivity with respect to the action of aqueous agencies. In order to render the enzymic substance accessible to the influence of such media, while retaining its specific capacity for action, we must choose a method which permits the lipase to remain in

[1] Contribution à l'ètude de la saponification des corps gras, Paris, 1906; see also Y. W. Yalander, Bio. Z. **36**, 435 (1911).

[2] H. **50**, 414 and particularly p. 423 (1906–07).

contact with fat during all the purification processes. Such a process was followed by Willstätter and Waldschmidt-Leitz [1] in the transformation of the enzyme in the form of a fat and water containing cream, whose preparation, already suggested in principle by Hoyer,[2] is based upon the separation of an aqueous emulsion of the oleaginous seeds. Because of its affinity to the oil of the seeds, the lipase is found in increased amount together with the latter in the compact, cream-like, surface layer thrown off by centrifuging. Preparates of considerably increased purity will be obtained if the enzyme in this form is subjected to the action of dilute aqueous reagents for the purpose of separating the substances ineffective as enzymes, as by means of alkaline, acid or salt solutions. Here too, however, a limit is placed on the prolonged use of such methods by the instability of the enzyme.

The success of the purification processes in so far as it is expressed in the analytically determined enzymic concentration of the dry preparate of the enzyme, is diminished moreover by losses in activity consequent upon the drying and de-fatting of the cream. Only by use of the mildest methods has it been possible to confine the observed losses of activity within reasonable limits. Thus it was necessary first to thoroughly dehydrate the enzymic cream in a moderately warm current of air and then from the residuary oil suspension to transform the active dry substance into the fat-free form by very gradual dilution with oil solvents. The most favorable enzymic concentration obtained was about 100-fold (Ph.L.W. = 5.23) that of the crude source materials (Ph.L.W. = 0.054). However, even by further application of the reagents used it was not found possible to obtain the enzyme free from protein substance.

For pancreatic lipase, which can be entirely freed from albuminous substances, the presence of these albuminous substances under natural conditions of action is of great importance with respect to the activity of the enzyme. It facilitates the contact between the enzyme and the substrate by the formation of " complex adsorbates." Plant lipase in the cytoplasm of the cell appears to be already attached to an insoluble, protein-like carrier, together with its substrate; but in order to bring it into action here a special alteration of the enzyme complex is required.

[1] Same citation as before, particularly p. 186.

[2] H. **50**, 414 and particularly p. 430 (1906–07).

Alteration During Germination.—This alteration of the enzyme complex takes place during the germination of the seed. It can also be caused artificially by the action of an animal protease, pepsin. The enzyme originally present in the seeds, which is characterized by an optimum *p*H of 4.7 to 5.0 and by its inactivity in neutral reaction, diminishes more and more in the course of germination or of the pepsin action, and the seeds then acquire an increasing capacity to saponify fats in a neutral and even in a weakly alkaline solution. The observed alteration in enzymic properties is to be ascribed to a hydrolytic decomposition of the protein components of the enzyme either through the action of the seed protease or pepsin. The theory which prevailed in the earlier literature,[1] according to which the formation of acids was required for the release of the lipatic functions, has not been confirmed.[2] The action of acid on the enzyme of the ungerminated seed leads rapidly to the formation of an addition product of lipase and acid, which is likewise capable of decomposing fats in neutral solution, but differs in its properties and in its activity powers from the products formed by means of proteolytic decomposition.

The transformation of lipase during germination, on the other hand, is marked by a rather gradual process; it corresponds to the gradual mobilization of the reserve fat of the seeds, which appears to be preceded by a certain hydrolysis of the seed proteins. But even in this transformation the enzyme is not carried over in soluble form and moreover its affinity to lower glycerides is not increased. The new form of the enzyme, "blasto-lipase," differs meanwhile from the original one, "spermato-lipase," in many other respects. Thus, its sensibility toward changes in the colloidal state is diminished, probably because of a diminution of the molecular complex. Likewise, its synthesizing properties are more strongly marked; they are still exhibited in very dilute glycerine. The organism of the growing plant evidently has here at its disposal a special power of regulation for the splitting of its high-valence reserve fats.

[1] Compare W. Connstein, E. Hoyer and H. Wartenberg, B. **35**, 3988 and particularly p. 4004 (1902).

[2] See R. Willstätter and E. Waldschmidt-Leitz, same citation as before, particularly pp. 163 and 205 (1923–24).

b. Chlorophyllase

Action.—Willstätter and Stoll [1] have found chlorophyllase to be present in the green leaves of all investigated classes of plants; it is always present associated with the green coloring matter of the leaf. Its action consists in the cleavage of the ester linkage of one of the three carboxyls of the chlorophyllide with the high molecular alcohol, phytol, $C_{20}H_{39}OH$, as it occurs in natural chlorophyll. This reaction takes place, for example, with chlorophyll in an aqueous acetone solution. (Powdered leaves, exhaustively freed from cholorophyll by means of alcohol, are used as the active enzyme material.) Nevertheless, in the presence of methyl or ethyl alcohols, the formation of free acid is not observed but a reversed esterification in which the phytol is replaced by ethyl or methyl alcohol. In the case of ethyl alcohol, the so-called crystallized chlorophyll or ethyl-chlorophyllid is formed.

The action of the chlorophyllase is confined with strict specificity to the chlorophyll itself and to its nearest derivative, its magnesium-free basic substance, phäophytin; the enzyme does not act on fats or waxes. The reaction course of chlorophyll cleavage appears to belong to an equation of the first order, in so far as its reaction velocity can be measured in the unfavorable circumstances which might be expected to prevail here; these circumstances are caused by the condition of the highly diluted enzyme, as well as by the complicated and practically unknown composition of the leaf extract.

Analytical Procedure.—The course of the hydrolysis of the chlorophyll is determined by a simple colorimetric method, based upon the acid nature of the free chlorophyllide formed; it is extracted with alkali from the reaction mixture carried over in ether. For the purpose of observing the alcoholysis of the leaf-coloring matter on the contrary, i.e., the exchange of phytol by alcohol, two special analytical processes are used. One is based on the determination of the phytol content remaining after the action of the enzyme, i.e., by complete saponification and weighing the isolated alcohol. The second is based on a determination of the "silver iodide number," i.e., the quantity of silver iodide obtained after splitting off the methoxyl or ethoxyl by means

[1] A. **378**, 18 (1910); **380**, 148 (1911).

of hydriodic acid. The reversal of the hydrolytic process, the esterification of the free carboxylic acid chlorophyllide with phytol, under the action of chlorophyllase has also been observed.

c. Tannase

Occurrence and Action.—Tannase, whose splitting action, with respect to tannin, has already been observed in mold fungi by Scheele, must be regarded as a specific esterase having a special affinity for the esters of phenol carboxylic acids. The latter, in the form of a depside compound, represent, according to Fischer, a characteristic structural type of the natural dyestuffs. Such depsides form a chain of several phenol carboxylic acids, in which the carboxyl group of the one is connected by an ester linkage with the phenolic hydroxyl group of another. Thus, tannase, which was first separated by Fernbach [1] and Pottevin [2] from the mold *aspergillus niger*, and which effects the hydrolysis of such depsidic linkages, has rendered valuable service, in the researches of Freudenberg,[3] for the constitutional elucidation of natural tanning materials. Meanwhile no one has yet attacked the problem as to whether the enzyme obtained by alcoholic precipitation from the autolytic juices of the fungus may be regarded as an integral esterase or whether it occurs associated with other ester-splitting enzymes. Likewise no data are found in the literature which would enable us to form a positive opinion as to whether the fungus always forms a number of esterases, or whether it is only in breeding upon a special tannin-rich nutrition media, whereby its tannase content is enriched, that a specific production of this esterase is predominant, similar to the phenomenon prevailing in the accustoming of yeast in the fermentation of galactose.

Kinetics.—Tannase has been more extensively investigated by Freudenberg and Vollbrecht,[4] who obtained the enzyme by precipitation with alcohol from the fungus bred upon myrobalans and autolyzed in a medium maintained constantly neutral. The simple substrate employed in these researches, gallic acid-methyl ester, should be suited to the measurement of kinetic reactions;

[1] C. R. **131**, 1214 (1900).

[2] C. R. **131**, 1215 (1900); **132**, 704 (1901).

[3] B. **52**, 177 (1919); **53**, 232, 953, 1728 (1920); **54**, 1698 (1921); **55**, 2420 (1922).

[4] H. **116**, 277 (1921).

however, the strong alteration of acidity caused by the formation of free gallic acid under the action of the enzyme, which was not excluded, has prevented a positive recognition of the reaction process. In this investigation by Freudenberg and Vollbrecht this reaction is supposed to follow a mono-molecular course. Moreover, for the same reasons, the *p*H dependence of tannase action has not yet been definitely recognized. The measurement of enzymic activity, which is expressed by the "cleavage value," depends upon the empirically determined relations between the enzyme quantity and the transformation.

d. Phosphatase, Sulfatase

Enzymes which effect the cleavage of organic phosphoric acid esters, e.g., the phosphoric ester of glycerine or of sugar, are widely distributed in the animal and plant kingdoms. Their separate existence and their difference from the ordinary esterases of animal tissues or of plant cells, however, is not yet to be regarded as certain. Proof of this can be expected only from their preparative isolation, which has as yet been neither accomplished nor even attempted. It is not directly probable that the animal organism or one-celled organisms as yeast will produce on occasions different enzymes for the hydrolysis of phosphoric acid esters of glycerine or of the various hexoses. When, therefore, we find numerous observations in the literature regarding the cleavage of such esters, e.g., by yeast or by animal organs, we need not refer these in all cases to different enzymic individuals. To these observations belong the reports concerning the hydrolysis of glycerine phosphoric acid by yeast [1] or by an enzyme of the intestinal mucous membrane,[2] and also the important observations of Harden and Young [3] and of Embden [4] concerning the synthesis and cleavage of fructose diphosphoric acid, the lactacidogene. This latter plays an important intermediary role in alcoholic fermentation and in the carbohydrate conversion in active muscle. The differentiation of a separate hexosephosphatese [5] from the

[1] C. Neuberg and L. Karczag, Bio. Z. **36**, 60 (1911).

[2] P. Grosser and J. Hussler, Bio. Z. **39**, 1 (1912).

[3] Proc. Roy. Soc. B. **82**, 321 (1910).

[4] See H. **93**, 1, 54, 124 (1914).

[5] See H. v. Euler and S. Kullberg, H. **74**, 15; **76**, 468 (1911); H. v. Euler and Y. Funke, H. **77**, 488; **79**, 375 (1912); H. P. Barendrecht, Bio. Z. **118**, 245 (1921).

hydrolysing enzyme, as well as the description of a specific saccharophosphatase [1] in yeast, is yet not to be accepted as positive.

The phosphatases also participate in the hydrolytic degradation of nucleic acids, since they are capable of rupturing the carbohydrate-phosphoric acid linkage in the nucleotides. Such an action, which is attributed by Levene and Medigreceanu [2] to special nucleotidases, is observed in the extracts of various animal organs, particularly the mucous membrane of the intestine.

Finally, the sulfatases may also be mentioned as belonging to the group of esterases. Their action was first described by Neuberg.[3] Characteristic of their action is the cleavage of the so-called ether sulfuric acids, the sulfuric acid esters of phenols, i.e., of products built up by the organism for the de-toxication and elimination of phenols introduced. Such enzymes are found in mold fungi such as *aspergillus oryzae*, and their action has been demonstrated by Neuberg in the muscle as well as in the kidney.

[1] See K. Djenab and C. Neuberg, Bio. Z. **82**, 391 (1912).
[2] J. Biol. Chem. **9**, 65, 389 (1911).
[3] Naturw. **12**, 797 (1924).

II. PROTEASES AND PEPTIDASES

All those enzymes which participate in the degradation of the proteins to their simplest structural units, the amino acids, are considered under the collective concept of proteolytic enzymes. The task of these enzymes consists in the rupture of acid amide linkages, the linkages —NH—CO—, with the formation of free amino and carboxyl groups.

Northrop [1] has undertaken and experimentally supported a division of the proteases according to a predominantly physicochemical point of view. This division is based on the differentiation of two types of proteolytic enzymes, according to whether they enter into reaction with the negatively or with the positively charged ions of the amphoteric proteins. To these a third type is added, whose action is referred to the action on the undissociated, electrically neutral protein molecules. However, serious objections are to be raised against such a classification, which is generally unfeasible, since sufficient consideration is not given to the chemical structures, which actually are of primary importance to the action of an enzyme. A rational classification of the proteolytic enzymes must be based on a knowledge and comparison of the special structural groupings to which the action of the enzyme relates. In the following treatments, the experimental attempts which have been made in this direction will be particularly emphasized.

DETERMINATION OF PROTEOLYTIC DEGRADATION

The numerous methods described in the literature for following the course of enzymic proteolysis can be divided into two principal classes, according to whether they are based on the determination of the unaltered substrate or upon the measurement of the decomposition products formed.

Methods Based on Determination of Unaltered Substrate.— To the first class belong the important earlier methods of deter-

[1] Naturw. **11**, 713 (1923).

mination by Ebstein and Grützner [1] as well as by Mett,[2] which consist in the dissolution of coagulated proteins. They are, however, of only historical significance, since they do not permit exact nor even comparable measurements. Furthermore, the objection is to be raised against some of the more recent processes, which determine the diminution of substrate quantity, that they ordinarily represent only a portion of the enzyme action. This is true, e.g., of the methods by Volhard,[3] by Gross [4] and those by Fuld and Levison,[5] according to which the quantity of the unattacked substrate, e.g., of casein or edestin, is determined by precipitation and weighing. It is also true of the newer method of determination by Rona and Kleinmann,[6] which depends on the nephelometric measurement of the undigested substrate after it is separated out. Also the older method of Grützner,[7] recently modified by Waldschmidt,[8] can lead only to more or less accurate evaluations. In this method the enzyme action is not determined on the basis of the amount of substrate which remains undissolved, but is determined colorimetrically on the basis of the amount carried over.

Physical Methods Based on Determination of Cleavage Products.—More important are those methods, which are based upon measurement of the cleavage products formed or upon the continuous observance of the alteration of definite properties of the reaction mixture. Thus the process of viscosity determination according to Fermi,[9] as employed by Palitzsch and Walbum [10] for the tryptic digestion of gelatine, and the measurement of electrical conductivity according to Henri and Bancels,[11] permit the study of a somewhat broader phase of proteolysis. However,

[1] Pflüg. Arch. **6**, 1 (1872).

[2] Arch. Physiol. 1894, p. 68.

[3] Münchner med. Wochenschr. 1903; p. 2129; 1907, p. 403; see also W. Löhlein, Hofm. Beitr. **7**, 120 (1905).

[4] Arch. exp. Pathol. u. Pharmakol. **58**, 157 (1906).

[5] Bio. Z. **6**, 473 (1907).

[6] Bio. Z. **140**, 461 (1923); H. Kleinmann and K. Asada, Klin. Woch. **3**, 572 (1924).

[7] Pflüg, Arch. **8**, 452 (1874); **144**, 545 (1912).

[8] Pflüg. Arch. **143**, 189 (1912).

[9] Archiv. für Hygiene, **12**, 238 (1891); **40**, 155 (1906).

[10] Bio. Z. **47**, 1 (1912).

[11] Soc. Biol. **55**, 563, 787, 788, 789 and 866 (1903); compare also W. M. Bayliss, J. of Physiol. **36**, 221 (1908).

their principles are not sufficiently general and the region of their applicability is too restricted, while at the same time they do not permit a definite comparison of the hydrolyses of different substrates. On the other hand, the use of polarimetric analyses, which determine the change in rotation during proteolytic processes, may possess a more general significance, particularly in simple cases. This is a process which, according to the example by Abderhalden and Koelker,[1] has been successfully used before only for following the hydrolysis of simple, optically active peptides. In its importance for the recognition and the solution of problems of structural-chemical and enzyme-chemical nature in the field of protein chemistry, polarimetric analysis may be compared to the four most important recent methods of proteolytic study.

Chemical Methods Based on Determination of Cleavage Products.—The important gas-analytical process of van Slyke [2] is to be counted among the four fundamental methods which permit a comprehension as to the definite chemical changes taking place during the enzymic degradation of the proteins. This method permits a volumetric analysis of the quantity of free amino groups on the basis of their reaction with nitrous acid, which results in the evolution of nitrogen. Another reliable method for the determination of basic amino groups is founded on titration procedure.[3] According to this method, the substance is titrated with 0.1N HCl in alcoholic solution after adding acetone to 90 per cent concentration. Naphthyl red or pentamethoxy triphenyl carbinol are used as indicators. The only amino acids to which the method is not applicable are those whose amino groups are very feebly basic (aromatic amino acids) or those whose acid groups are very strongly acidic (sulfonic acids, taurine). Ordinary enzyme buffers apparently do not affect the titration.

The two other processes have to do with the determination of the carboxyls liberated during proteolysis. These are the much-used formol titration method of Sörensen,[4] and the alkalimetric determination of amino acids and peptides in alcoholic

[1] H. **51**, 294 (1907).
[2] B. **43**, 3170 (1910).
[3] K. Linderström-Lang, H. **173**, 32 (1928), ibid. **174** 275 (1928).
[4] Bio. Z. **7**, 43 (1907); **25**, 1 (1910).

solution, according to Willstätter and Waldschmidt-Leitz.[1] Formol titrations depend on the covering up of the basic amino groups of amino acids, peptides and proteins by formaldehyde and on the consequent development of the acid function of the associated carboxyl groups of these amphoteric substances. These titrations make possible a very intimate comprehension, with a few exceptions, of the course of proteolytic processes, in so far as these are expressed in the rupture of acid amide linkages, i.e., in the liberation of amino and carboxyl groups. However, their applicability fails entirely or at least the results are only circumstantial if ammonia salts or phosphates occur in the proteolytic degradation mixture; the latter are sometimes present as *p*H regulators.

Alkalimetric determinations in alcoholic solution, according to the method of Willstätter and Waldschmidt-Leitz, appear to be of even more general applicability than formol titrations. The addition of alcohol suppresses the basic properties of the amino groups, develops the acidity of the carboxyls and ensures exact results even in the presence of ammonium salts or phosphates. This procedure also makes possible a differentiation between the proportions of the amino acids and the peptides on the basis of their acidity, whereas, the stepwise formol titration of Henriques and Sörensen[2] permits only an approximation of these relations. The acid properties of the peptide carboxyls are more strongly pronounced, their amount being expressed completely in 50 per cent alcohol, while that of amino acids is exhibited only in concentrations of over 90 per cent.

However, the use of either of these methods of investigation alone is not sufficient for the more intimate knowledge of the processes which take part in the degradation of proteins. We must rather first obtain, through a combined and comparative investigation of the most varied analytical methods, a closer insight into the manner in which the individual proteases work, and therewith into the structural problems of protein chemistry; too little has as yet been done along this line.

[1] B. **54**, 2988 (1921).

[2] H. **63**, 27 (1909); V. Henriques and J. K. Gjaldbaek, H. **71**, 485; **75**, 363 (1911).

1. TRUE PROTEASES

a. Pepsin, Lab

Formation.—The protein digestive action of the stomach secretion was first recognized as an enzymic process by Schwann.[1] The enzyme, which he termed pepsin, is found chiefly in the mucous membrane of the stomach, and is secreted by the latter together with the stomach juices. The velocity of this secretion process, which sets in upon ingestion of albuminous food, follows the mono-molecular time law, as calculated by Herzog [2] on the basis of experiments made by Pawlow.[3] That is, it follows the equation in which the velocity of the secretion $k = \frac{1}{t} ln \frac{a}{a-x}$, when x represents the enzyme quantities excreted during the time t, and a the total amount excreted during the experiment. The hormonal inauguration of the secretion process is to be ascribed, according to the researches of Frouin [4] and others, to an internal secretorial substance contained in the membrane itself, the gastrin. Experiments have shown that the action of this substance can be manifested from the blood stream.

Supposed Inactive Pre-Stage.—The numerous statements, especially in the earlier literature, with respect to the secretion of the enzyme in the form of an inactive pre-stage, pepsinogen or pro-pepsin, are no longer regarded as correct. In support of this assumption some of the supposedly essential differences between the inactive and the active form of pepsin are advanced, such as varying stability against alkali. However, the more logical explanation is that the distinctions observed are to be attributed to *p*H effects, particularly in that an activation of the zymogen by acid has been described. The differences of stability mentioned might be attributed possibly to different amounts of associated substances in the enzyme solutions compared.

Hydrogen-Ion Dependence.—The dependence of pepsin action on acidity, although observed much earlier by others, was first basically studied by Sörensen.[5] In this work it has been found

[1] Müller's Archiv. 1836, p. 90.

[2] H. **41**, 425 (1904).

[3] Die Arbeit der Verdauungsdrüsen. Wiesbaden 1898.

[4] Soc. Biol. **57**, 887 (1905).

[5] Bio. Z. **21**, 131 and particularly p. 288 (1909); See also L. Michaelis and H. Davidsohn, Bio. Z. **28**, 1 (1910).

that the optimal action of the enzyme, measured in the digestion on egg albumin, corresponds to a strongly acid reaction. The optimal *p*H is about 1.5 if the duration of the experiment is very brief; upon more prolonged or extensive transformation, however, there is noted a considerable expansion in the region of optimal activity in the direction of lower acidity. The cause of this change has not yet been recognized. According to Northrop [1] the dependence of the action of pepsin is influenced, like that of other proteolytic enzymes, by the ionization state of the substrates used. Since, according to this view, the action of pepsin is to be attributed to its reaction with the protein kations, then its *p*H optimum is displaced at the same time as the iso-electric point of the substrate. The results obtained in the investigations of Northrop for the *p*H dependence of pepsin and of trypsin are graphically represented in Fig. 10. For example, the optimum *p*H for the hydrolysis of

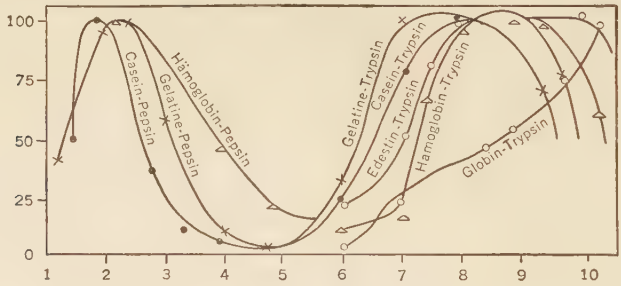


FIG. 10.—Activity-*p*H curves of pepsin and trypsin with various substrates.

casein by pepsin is 1.8 and that for haemoglobin and gelatin 2.2. The observed differences, which are expressed still more markedly by the form of the activity-*p*H curves than in the position of the optima, might also be conditioned in part by a difference in the methods of determination employed.

Kinetics.—Schütz [2] first established the law named for him with respect to the reaction course of the hydrolysis of egg albumen by pepsin, according to which the quantities of peptone formed are found to be directly proportional to the square roots of the enzyme quantities employed. The relations noted, which are given in Table XXVII, have been later confirmed by Schütz [3] through another method of determination.

[1] J. Gen. Physiol. **1**, 607 (1919); **3**, 211 (1920); **5**, 263 (1922).
[2] H. **9**, 577 (1855).
[3] H. **30**, 1 (1900).

TABLE XXVII

PEPSIN QUANTITY AND TRANSFORMATION

(*According to Schütz*)

Pepsin Quantity	Rotation (′)	
	Observed	Calculated
1	9.40	10.8
4	20.6	21.6
9	32.3	32.4
16	45.4	43.2
25	55.2	54.1
36	65.0	64.9
49	76.0	75.7
64	85.3	86.5

The validity of the Schütz law for action of the pepsin has also been verified by other researches using the most varied methods of determination, for example, in the works by Sjöqvist,[1] by Spriggs,[2] by Küttner[3] or by Herzog and Margolis.[4] Arrhenius,[5] who has discussed the theoretical principles underlying this law and has cited a relevant example from the field of non-enzymic catalysis, has sought to explain the experimental results obtained by Schütz and by Sjöqvist through the aid of other formulas. On the other hand, Abderhalden and Steinbeck[6] find that for the dissolution of solid, denatured proteins, the product of the enzyme quantity and the time for equal transformations is approximately constant. However, the present knowledge as to the mechanism of this process is too limited to permit any conclusive opinions.

Specificity and Mode of Action.—In general, all genuine proteins, aside from the protamines, are hydrolysed by pepsin, in so

[1] Skand. Arch. f. Physiol. **5**, 317 (1895).

[2] H. **35**, 465 (1902).

[3] H. **52**, 63 (1907).

[4] H. **60**, 298 (1909).

[5] Immunochemie, Leipzig, 1907, and particularly p. 53.

[6] H. **68**, 297 (1910); see also H. **71**, 315 and 339 (1911); Fermentf. **6**, 248 (1922); **7**, 61 (1923).

far as they are not to be regarded, like the protenoids, the substances of the animal skeleton, as generally unattackable by enzymes. But the velocity with which the degradation of the individual proteins takes place depends upon their nature and their composition in varying degree. The phenomenon that a previous coagulation renders some proteins more easily hydrolysable by pepsin and others more difficultly hydrolysable in not to be explained by a change in the colloidal state, but rather by a chemico-structural change in the molecule of the proteins, which either favors or blocks the attachment of the enzyme.

According to the researches of Fischer and Abderhalden,[1] it is regarded as certain that pepsin is ordinarily not capable of rupturing acid amide linkages in polypeptide chains, although the selection of the peptides tested may have been insufficient. The same thing is true of the cleavage of simple 2, 5-diketopiperazines, according to the results obtained by Abderhalden,[2] as well as by Waldschmidt-Leitz and Schäffner.[3]

It is true that according to the attitudes expressed in the literature and on the basis of some recent observations, it may appear dubious as to whether or not it is possible to ascribe the specific action of pepsin to definite alterations in the chemical structure of the proteins, and to comprehend these by chemical methods. Oppenheimer in his "Die Fermente und ihre Wirkungen"[4] suggests that the action of pepsin consists in the colloidal disaggregation of the protein and in the dissolution of residual valences which hold the complex polymer together. A distinction is made between such disaggregating ferments and true hydrolytic ferments. Nevertheless a number of recent observations positively demonstrate that the action of the enzyme is expressed in definite chemical transformations. To these belong the results obtained by Henriques and Gjaldbaek[5] with respect to the development of formol-titratable nitrogen, the observations of Frankel[6] concerning an increase of free amino groups, and also the finding of Felix[7] with respect to the increase of methylatable

[1] H. **46**, 52 (1905).
[2] H. **55**, 384 (1908); E. Abderhalden and K. Goto, Fermentf. **7**, 691 (1923).
[3] B. **58**, 1356 (1925).
[4] 5. Aufl., Leipzig, 1925, 2. Bd., p. 812.
[5] H. **75**, 362; **83** (1913).
[6] J. Biol. Chem. **26**, 31 (1916).
[7] H. **146**, 103 (1925).

nitrogen and the formation of free lysine during the action on histone. These observations have been verified and extended by comparative measurements made by Edlbacher,[1] by Waldschmidt-Leitz and Simons[2] and by Waldschmidt-Leitz and Künstner.[3] Such studies have involved the relation in which amino groups and carboxyls are liberated during the course of peptic hydrolysis. Whereas, according to Edlbacher and according to Steudel,[4] the number of carboxyls formed exhibits a greater increase than the quantity of methylatable N as a measure of the free amino groups, Waldschmidt-Leitz and Simons were able to show that in peptic digestion of a number of proteins, the free amino nitrogen, measured by Van Slyke's method, increases at the same rate as the formation of free carboxyls, and that consequently the action of the pepsin consists in the rupture of acid amide linkages.

More recent investigations by Felix and Harteneck,[5] with histone and by Weber and Gesenius[6] with casein confirm the equivalence of NH_2 and COOH. Waldschmidt-Leitz and Künstner further demonstrate this constant relation of NH_2 and COOH formation with four typical proteins (egg albumin, casein, gelatin and histone) and point out the cause of the divergent observations which have been reported. These divergencies in the direction of a stronger carboxyl increase are shown to be due to an incomplete determination of the intrinsic acidity of the unaltered substrate in the initial alkalimetric titration. This same cause might also be responsible for the variations noted by Waldschmidt-Leitz and Simons in the hydrolysis of certain cereal proteins. Their unfavorable solubility behavior, however, does not permit a positive removal of the analytical sources of error.

In the investigation by Waldschmidt-Leitz and Künstner, acidity is determined by titrating the substrate (casein, egg albumin or gelatin) with 0.2N alkali against thymolphthalein. Upon appearance of a weak blue coloration, boiling alcohol is added and the titration continued to the end. The proteins are thrown out of solution by the alcohol in such a finely dispersed

[1] H. **107**, 52 and particularly p. 67 (1919); **108**, 287 (1919–20).

[2] H. **156**, 114 (1926).

[3] H. **171**, 70 (1927).

[4] J. Ellinghaus and A. Gottschalk, H. **154**, 21 and 198 (1926); H. Steudel and J. Ellinghaus, H. **166**, 84 (1927).

[5] H. **165**, 103 (1927).

[6] Biochem. Z. **187**, 410 and particularly 429 (1927).

state that the total acidity of the material can be determined without error.

These results have been substantiated by Sörensen and Katschioni-Walther,[1] who also find the ratio 1:1 for carboxyl and amino groups liberated during the peptic digestion of casein, gelatin, and gliadin. Carboxyl groups were determined by formol titration and by Willstätter's titration in alcohol solution. Amino groups were determined by the Van Slyke method and by Linderström-Lang's titration in acetone solution with naphthyl red as indicator.

That the action of pepsin is expressed by the rupture of —CO—NH— linkages is further confirmed by the observations that the capacities of the various proteolytic enzymes for rupturing such linkages stand in simple, whole-numbered ratios. This has been demonstrated by Waldschmidt-Leitz and Künstner[2] in the case of histone and by Waldschmidt-Leitz and Simons[3] in the case of casein. Table XXVIII illustrates the relative capacities of th eenzymes participating in the degradation of histone.

TABLE XXVIII

HYDROLYSIS OF THYMUS HISTONE BY PEPSIN, TRYPSIN, TRYPSIN-KINASE AND EREPSIN

Hydrolysis of 0.20 g. Histone sulfate; capacity unit chosen corresponds to 0.70 ccm. 0.2 *n*-COOH

Sequential Order of Enzymes	Increase (Ccm. 0.2*n*)		Capacity
	COOH	NH_2	
Pepsin	0.75	0.76	1
Trypsin	0.76	0.75	1
Trypsin-Kinase	2.75	2.75	4
Erepsin	(2.70)	(2.70)	(4)
	6.96	6.96	10

The action of pepsin, accordingly, consists in the rupture of about one-tenth of the linkages which are ruptured upon complete

[1] H. **174**, 251 (1928). [2] H. **171**, 290 (1927). [3] H. **156**, 99 (1926).

hydrolysis. It is also shown by further experiments that its action can be substituted by other enzymes. Thus, the complete splitting of histone can also be accomplished by the combined action of trypsin-kinase and erepsin without the action of pepsin. The degradation is considered to be complete in that the same degree of hydrolysis is obtained with the above combinations of enzymes and also with acids. In this case the trypsin-kinase acting first ruptures 60 per cent of the total —NH—CO— linkages broken and the intestinal erepsin 36 per cent. Table XXIX represents the action on histone in the same way as in the previous table.

TABLE XXIX

HYDROLYSIS OF THYMUS HISTONE BY TRYPSIN-KINASE AND INTESTINAL EREPSIN

(Same conditions as Table XXVIII)

Sequential Order	Increase (Ccm. 0.2*n*)		Capacity
	COOH	NH_2	
Trypsin-kinase	4.30	4.30	6
Intestinal erepsin	2.55	2.50	4
	6.85	6.80	10

Preparative Purification.—The results of the preparative purication of pepsin, as indicated in the earlier researches, are rather difficult to evaluate. Extensive purification by modern enzymic methods has not yet been successful, although it was with this enzyme that the first observations of enzyme adsorption were made. In characterising the state of preparative research it may suffice here to say that pepsin has been reported on the one hand in phosphorus free preparates [1] by working with the stomach secretion, and on the other hand in protein-free preparates [2] by separation from the mucous membrane.

[1] Compare C. A. Pekelharing, H. **35**, 8 (1902); **75**, 282 (1911); W. E. Ringer, H. **95**, 195 (1915).

[2] Compare C. Sundberg, H. **9**, 319 (1885); P. Schrumpf, Hofm. Beitr. **6**, 396 (1905).

Plastein Formation.—The apparent re-synthesis of a protein-like material was first reported by Danilewski in 1866, who observed the formation of a precipitate when stomach extract was added to a concentrated solution of the products of peptic hydrolysis. The phenomenon was ascribed to enzyme action because the precipitation did not take place if the stomach extract was previously heated to 100° C. Sawjalow [1] applied the term "plastein formation." According to his experiments it was necessary for the albuminose fractions to be present; the "synthesis" was not accomplished when various fractions of Witte's peptone were used, although large quantities of the product were formed when they were recombined to make up the original mixture. Taylor [2] and Robertson [3] have reported synthesis of protein by means of trypsin and pepsin.

As a criterion for this synthesis, the above investigators took the formation of a difficultly soluble product from the more readily soluble degradation products. In 1911 and 1912, however, Henriques and Gjaldbäk,[4] pointed out that there was a disappearance of part of the free amino nitrogen during the process. This was considered as evidence that the complexity of the plastein molecule was greater than that of the substances from which it was derived. The assumption here is that as the increase of the free amino N of a protein conversion has often been taken as a measure of the degree and rate of hydrolysis, a decrease in the ratio of free amino to total nitrogen may justifiably be interpreted as proof of synthesis.

The above observation as to the decrease in amino nitrogen has been confirmed by Wasteneys and Borsook [5] and by Rona and Chrometzka.[6] Wasteneys and Borsook consider that the reaction is reversible and subject to the mass action law. Rona and Chrometzka, however, contest the assumption that any synthetic action takes place. Their conclusions are based on more extensive chemico-analytical characterizations. They have followed the course of enzyme action by determining the change in amino

[1] Arch. ges. Physiol. **85**, 171 (1901).
[2] J. Biol. Chem. **3**, 87 (1907); ibid, **5**, 381 (1908–09).
[3] J. Biol. Chem. **3**, 95 (1907); **5**, 493 (1908–09).
[4] H. **71**, 485 (1911); ibid, **81**, 439 (1912).
[5] J. Biol. Chem. **62**, 15, 633 (1924–25); ibid, **63**, 563 (1925).
[6] H. **189**, 249 (1927).

nitrogen and in the carboxyl groups. Amino nitrogen was determined by the Van Slyke method and carboxyls by the Willstätter method. By the action of pepsin on concentrated, clear, water solutions of protein degradation products they found a consistent decrease of amino nitrogen in the insoluble products formed. However, the amount of carboxyls remained practically constant or else increased slightly. Also by demonstrating the formation of ammonia, the disappearance of amino nitrogen is shown to be due to deaminization and not to further combinations. On the basis of such facts, they consider that no synthesis takes place and that the insoluble product is a degradation product even though less soluble.

Milk Coagulative Action.—There should be mentioned here another important property, recognized in earlier times, of the extracts and secretions of the mucous membrane of the animal stomach, i.e., the capacity to coagulate milk, which has often been ascribed to the action of a special proteolytic enzyme, lab. This process, which occurs only in the presence of soluble salts of earth alkalies, depends upon the enzymic transformation of the chief albuminous substance in milk, caseinogen, into casein, and upon the precipitation of the latter in the form of an insoluble salt. However, it is not specific for the stomach enzyme; it is also observed in the action of other proteases, as in the trypsin of the pancreas or the papain from the melon tree. The recognition of this process has contributed significantly to the contravention of a distinction of a special labic enzyme besides the pepsin of the stomach mucous membrane and to the conception of milk albumin coagulation as only the first phase of its proteolytic degradation. This is also supported by the fact that flocculated casein is always subject to further hydrolytic dissolution. The subject was taken up in particular in the investigations by Hammarsten.[1] The earlier reports of Hammarsten, which announced a separation of pepsin and lab, and the preparation of pepsin-free solutions of the lab enzyme, have not been confirmed.

According to the researches of Pawlow and his school [2] as well as the more recent researches of Hammarsten [3] the enzyme

[1] See R. Malys Jahresbericht über die Fortschritte der Tierchemie, 2. Band für 1872, p. 118 (1874).

[2] H. **42**, 415 (1904); **46**, 307 (1905); **54**, 32 (1907); **55**, 84 (1908).

[3] H. **56**, 18 (1908).

solution in these cases is not free from pepsin, but only free from pepsin action; it contains inactive pepsin. The question as to the distinction between lab and pepsin, which has been studied a great deal, is to be answered partially, on the basis of our more recent knowledge, by the fact that an enzyme with pronounced labic action occurs only in the stomach mucous membrane of young animals, e.g., of calves; whereas, Hammarsten [1] in making enzyme preparations from more mature animals, in which the pepsin action has increased and the lab action decreased, notes a changed form of the true lab or chimosin, the "parachimosin." No positive proofs against the identity of the two enzymes are given by these findings; such proofs can be reached only by a total separation of their two respective activities. The observed variations of the individual enzyme actions, which correspond to the physiological requirements, do not need to be explained by the distinction of two different enzymes. They recall the specific establishment and accustoming of the zymase complex of yeast to non-fermentable substrates, which are to be ascribed, not to the formation of a new enzyme, but to a reversion and alteration of the enzymic affinity.

Proteases in the Spleen.—An attempt has been made by Hedin [2] to differentiate two types of protein-splitting enzymes in the spleen according to their distinctive reaction optima. That is, it was assumed that extracts from the spleen contained an α-lienoprotease, acting in alkaline reaction, a β-lienoprotease, acting in acid reaction, and a peptidase of the erepsin type; the proteases were considered to be identical in their specificity relations. Later investigations by Waldschmidt-Leitz and Deutsch,[3] based on more exact methods for differentiating proteases and ereptases, have shown these assumptions to be entirely without basis.

The spleen has been shown to contain a protease acting optimally at pH 4 and a peptidase acting optimally at pH 8, the quantity of peptidase being predominant. Dernby [4] has also reported the optimum action of the peptidase or ereptase of the

[1] See H. **121**, 261 (1922); **130**, 55 (1923).

[2] S. G. Hedin and S. Rowland, H. **32**, 341, 531 (1901); S. G. Hedin, J. Physiol. **30**, 155 (1904); Biochem. J. **2**, 112 (1906–07); J. Biol. Chem. **54**, 177 (1922).

[3] H. **167**, 285 (1927).

[4] J. Biol. Chem. **35**, 179 (1918).

spleen to be about *p*H 8.0. Optimum protease activity is exhibited at only one point and not at two points as was supposed by Hedin. The supposition that there was a protease acting at about *p*H 8 was due to the presence of considerable quantities of ereptase. The method of separation by adsorption, developed by Waldschmidt-Leitz and Grassmann, was effective in successfully obtaining an erepsin-free protease and a protease-free erepsin. Nevertheless, the increase in purity-degree was comparatively unsatisfactory. This was particularly true in the case of the protease, which was relatively unstable and present in only small amounts.

b. Trypsin

Occurrence.—Trypsin, the action of which was first closely studied by Kühne,[1] is the principal proteolytic enzyme of the pancreas from which it is secreted with the pancreas juice into the intestine. The separation of this secretion takes place under normal conditions, due to the influence of a hormone, secretin, which is produced in the intestinal mucosa and carried to the pancreas by the blood stream.

Hydrogen-Ion Dependence.—The reaction of the pancreatic secretion, which is weakly alkaline, corresponds to the optimal region of action of the tryptic enzyme; a series of researches have been devoted to the determination of this region of optimum activity. Whereas, the first exact investigation of the *p*H dependence of trypsin action by Michaelis and Davidsohn [2] with peptone as substrate gave an optimum of $p\mathrm{H} = 8.0$, Palitzsch and Walbum [3] have found far more alkaline reactions to be optimal, e.g., $p\mathrm{H} = 9.9$ at 30° C., and Ringer [4] concluded from his researches that trypsin does not exhibit a definite region of optimum activity but that enzymic action increases steadily with increasing alkalinity. The latter results are to be attributed, however, merely to a disregard of the action of alkali on the substrate, since control experiments were not carried out with the buffers alone. According to Waldschmidt-Leitz,[5] the optimum of gelatin cleavage at *p*H 8.2—8.7 agrees with the results obtained by Northrop [6] according to whose

[1] Virch. Arch. **39**, 130 (1867).
[2] Bio. Z. **36**, 280 (1911).
[3] Bio. Z. **47**, 1 (1912).
[4] H. **116**, 107 (1921); **124**, 171 (1922–23).
[5] H. **132**, 181 and particularly p. 196 (1923–24).
[6] J. Gen. Physiol. **5**, 263 (1922–23).

researches, which show the dependence of the activity-*p*H curve on the nature of the substrate, a differing electro-chemical nature must be assumed for the individual ferment substrate compounds (compare the treatment in the foregoing section).

Kinetics.—The researches reported in the earlier literature, as might be expected, furnish no simple principles governing the complicated course of tryptic protein hydrolysis. Further, the proportionality between enzyme quantity and reaction velocity ordinarily holds good only for the transformation of small amounts. In the case of trypsin, Northrop [1] recently made a searching examination of the question of the formation of enzyme-substrate compounds and of the inhibiting action of the reaction products. While, according to these investigations, the linkage of the trypsin to the enzymic fission products, which is reversible, may be described by the mass action law, the attachment of the enzyme to its substrates, the formation of the enzyme substrate compounds, does not come under this concept. This conclusion is reached through the independence of inhibition by fission products, as well as from the researches regarding the influence of the substrate concentration upon the reaction velocity and upon the simultaneous hydrolysis of several substrates. The assumption of Northrop, according to which an intermediary enzyme-substrate compound is unnecessary for the action of proteolytic enzymes, has not been confirmed and evidence has been presented which would tend to discredit such an assumption. It is to be expected that comparative studies of tryptic hydrolysis by various analytical methods will result in a more profound knowledge of the kinetics involved.

Activation of Enterokinase.—It is an old view, going back to the observations of Kühne [2] and of Heidenhain [3] that the pancreas trypsin is produced from the gland, not in a finished state, but in the form of an inactive pre-stage. This appeared to acquire a confirmation in the fundamental finding of Schepowalnikow [4] regarding the specific influence of the intestinal juice upon the activation of the pancreatic secretion. It is possible to demonstrate that pure pancreatic fistula juice, completely inactive in the

[1] J. Gen. Physiol. **4**, 266 (1921–22); **5**, 487 (1922); **6**, (1923–24).

[2] Virch. Arch. **39**, 130 (1867).

[3] Pflüg. Arch. **10**, 557 and particularly p. 587 (1875).

[4] Inaugural Diss. St. Petersburg, 1899; Referat in Malys Jahresbericht über die Fortschritte der Tierchemie **29**, 378 (1899).

hydrolysis of genuine proteins, can be intensely activated by the addition of small amounts of intestinal juice. The action of the activator, which was termed enterokinase, was believed to be an enzymic transformation of the trypsin from its zymogen. The observations that the activation required time and that the activator was unstable were both regarded as decisive proof of its enzymic nature. This concept, which remained predominant in the literature,[1] has proved to be untenable. From an investigation by Waldschmidt-Leitz,[2] it was shown that the reaction between enterokinase and trypsin is based on stoichiometric principles, and that it likewise ceases after a definite, brief period of activity. An easily dissociated compound of enzyme and activator is formed, whose decomposition can be effected by adsorption methods. This fact is incompatible with the assumption of an enzymic transformation of trypsinogen into trypsin, especially since the enzyme and activator can be successfully separated by a simple preparative process, and the already maximally activated trypsin transformed anew into the inactive form. Furthermore, Waldschmidt-Leitz and Harteneck [3] have shown that in certain cases trypsin is capable of performing appreciable proteolytic degradation without the presence of an activator, e.g., in the action upon protamine or even peptone, and that, therefore, enterokinase must be regarded only as an assisting agent in the attack upon definite structures, particularly those present in the high-molecular proteins.

Spontaneous Formation of Enterokinase.—Another type of activation, the so-called spontaneous activation, already observed by Heidenhain [4] is frequently differentiated from the activation of trypsin by enterokinase, which is specific and which cannot be replaced by other agents, e.g., by lime salts or by gall salts, as was formerly believed. The former makes its appearance upon storage of the pancreas and its aqueous solutions or secretion and has been explained by an enzymic formation of trypsin. It has been found, however, that we are concerned here with the formation of an activator from the gland substance itself, which coin-

[1] Compare W. M. Bayliss and E. H. Starling, J. Physiol. **30**, 61 (1904); **32**, 129 (1905).

[2] H. **132**, 181 (1923–24).

[3] H. **149**, 203 and particularly p. 207 (1925).

[4] Pflüg. Arch. **10**, 557 (1875).

cides with the enterokinase in its behavior, e.g., in its adsorption behavior and in its characteristic properties. At one time, by means of adsorption on alumina, the activator was successfully withdrawn from spontaneously activated trypsin, in the same way as was carried out with the compound of trypsin and enterokinase; the activator was then detectable in the elution of the alumina adsorbates.[1] Furthermore, the use of this adsorption process with non-activated gland extracts has permitted the separation of the activator in the form of its pre-stage, "pro-kinase," from the trypsin, and has permitted the study of its formation independent of the presence of trypsin.

The determination of the converting power of the activator produced from the pancreas gland has shown that it is identical with enterokinase.[2] Furthermore, its formation, which depends upon the enzymic process, must be regarded as specific for the pancreas—it is not found in other animal organs. In the secretion of the pancreas also the trypsin is accompanied by the pre-stage of the activator, whose transformation into the finished kinase does not occur in the gland itself, as a protection against self-digestion, it is rather completed only in the cells of the intestinal mucous membrane. Normal secretions of the intestine do not contain any enterokinase and the content of the intestinal mucous membrane is dependent upon the entry of pancreatic secretions, in which the activator is found in the form of an inactive pre-stage. After removal of the pancreas no kinase or at least only an inappreciable amount appears in the intestinal mucous membrane even after several days.[3]

Preparation and Properties of Enterokinase.—From the information gained in regard to the nature of enterokinase action, the task of attempting the preparative purification of the activator and describing its properties at once arose. The method of purification followed in the research by Waldschmidt-Leitz [4] which employs a number of precipitations and adsorptions the control of which is based on a preliminary determination of the activator, has yielded in the best examples something like a 100-fold increase of concentration of the enterokinase from the dried intestinal

[1] E. Waldschmidt-Leitz H. **132**, 181, and particularly p. 191 (1923–24).
[2] E. Waldschmidt-Leitz, and A. Harteneck, H. **149**, 221 (1925).
[3] E. Waldschmidt-Leitz and J. Waldschmidt-Graser, **166**, 247 (1927).
[4] H. **142**, 217 (1924–25).

mucous membrane. (The preliminary estimation of the activator was accomplished by determining its action on a given quantity of pancreas.) The noteworthy fact was thereby established that the enterokinase, whose action was recognized to be of a non-enzymic nature, recalled enzymes in many of its properties. This was true of its capacity to form association products with concomitant substances, which as in the case of enzymes, distort the true behavior of the substance. Or it also holds good for the observed decrease of the stability of the activator in a purer solution and for its ability to be stabilized by glycerine. Furthermore, the temperature sensitivity of the activator, which, regardless of the degree of purity, undergoes a sudden destruction even at 50° C. may point to a labile enzyme-like structure.

Observations as to the adsorption behaviors of enterokinase, trypsin and trypsin-kinase have permitted some qualitative conclusions as to the linkages involved in certain special cases.[1] It has been shown that when enterokinase is adsorbed on alumina gel its capacity for activating trypsin is completely removed. That is, the trypsin binding group of enterokinase is manifestly involved in its adsorption on alumina. On the other hand, the activity of trypsin-kinase is exhibited quantitatively when in an adsorbed condition on alumina gel. The protein binding group, accordingly, is not involved here. Also trypsin and trypsin-kinase are adsorbed to the same quantitative degree by alumina gel, indicating that the same linkage between the enzyme and the adsorbent is involved in both cases.

Measurement of Trypsin Action.—Processes for the measurement of trypsin quantities have been based by Northrop [2] on the liquefaction of gelatin and by Willstätter and Persiel [3] on the increase of acidity during gelatin cleavage in alcoholic solution. Methods such as those of Willstätter and Persiel merit preferment over the former as well as over the earlier methods of determination, since they take into consideration the influence of the degree of activation of the enzyme and attempt its compensating activation with enterokinase. Other essential improvements which mark this analytical process depend on the finding that the sudden cessation of protein cleavage, which is caused by the strongly

[1] E. Waldschmidt-Leitz and K. Linderstrøm-Lang, H. **166**, 227 (1927).
[2] J. Gen. Physiol. **5**, 487 (1922.)
[3] H. **142**, 245 (1924–25).

inhibiting action of the cleavage products, requires the measurement of very small quantities of enzyme with large quantities of the substrate. The relations obtained between the enzyme quantity and the degree of cleavage have led to the establishment of a provisional measure for the amount of trypsin, the " trypsin unit (T.E.) " which has guided the preparative isolation of the enzyme.

Later findings have suggested the use of casein as a substrate for trypsin action.[1] For one thing, casein has been shown to be a specific substrate for trypsin + enterokinase, being attacked neither by kinase-free trypsin nor by erepsin. The analytical procedure, further, is simpler with casein. It does not form lumpy masses upon addition of alcohol, as does gelatin; this necessarily obviates a difficulty usually present when titrating gelatin reaction mixtures. A trypsin unit (T.E.) based on casein hydrolysis designates that enzyme quantity which effects a hydrolysis corresponding to 1.05 ccm. of 0.2 n—KOH under the given conditions. This unit is about equivalent to that used with gelatin.

Preparation of Trypsin.—It has been found desirable in the preparative production of trypsin to dehydrate and de-fat the source material, e.g., the pancreas of swine, immediately after the animal is slaughtered, as has been described in the discussion of pancreas lipase. The enzyme is thus obtained in a non-activated form, and the formation of inhibiting bodies, which appear at the beginning of the decomposition of the gland tissue and which affect the accuracy of the trypsin determination, is thereby avoided. The enzyme, which is extracted with good yields from the dried gland tissue with aqueous media or with glycerine, is associated in these solutions with the fat-, the starch- and the peptide-cleaving enzyme, as well as with the pre-stage of the enterokinase. Its separation from these is based on the preponderance of its basic properties, which enable us, first to extract, by adsorption with alumina, the lipase [2] and next erepsin [3] and pro-

[1] R. Willstätter, E. Waldschmidt-Leitz, S. Duñaiturria and G. Künstner, H. **161**, 191 (1926).

[2] R. Willstätter and E. Waldschmidt-Leitz, H. **125**, 132 and particularly p. 142 (1922–23).

[3] E. Waldschmidt-Leitz, and A. Harteneck, H. **147**, 286 and particularly p. 301 (1925).

kinase [1] from the solutions, while the main quantity of the trypsin, together with the starch-cleaving enzyme, remains behind in the mother liquor of the adsorption. Its separation is finally realized by a change of polarity in the adsorbent, i.e., the tryptic enzyme is taken out of the solution while kaolin and the amylase is left behind.[2] The trypsin is thus obtained in integral enzymic solutions, as has also been confirmed by more recent experiments of Willstätter, Waldschmidt-Leitz, Duñaiturria and Künstner.[3]

In the latter experiments the relative distribution of the enzymes has been followed by more quantitative controls. For example, the pancreas extract is brought to *p*H 4.7, alumina gel added twice, centrifuged off each time, and the mother liquor neutralized. The solution is found to contain 97 per cent of the original trypsin, 0.6 per cent of the lipase and 40 per cent of the amylase. The separation of the lipase accordingly is complete, whereas the amylase probably suffered considerable destruction in the acid solution. The mother liquor, now free from lipase, is treated four times with kaolin and the liquor separated each time by centrifuging. The combined adsorbates are then elutriated with basic ammonium phosphate and the resulting elution neutralized with acetic acid. No tryptic action is found in the mother liquor of this adsorption, whereas the elution contains about 75 per cent of the tryptic action of the original extract and practically no amylase activity.

Experiments show that this indifference of the trypsin toward adsorption by alumina in acid solution holds only for the stabile gel $Al(OH)_3$, designated as C_γ, whereas, the trypsin is completely adsorbed by using an insufficiently aged alumina preparate, which contains varying quantities of the unstable β modification. The presence of this modification must be strictly excluded in order to carry out successfully the above separation procedure.

In the above preparation, the trypsin is obtained not entirely free from its activator; that is, the resulting product is a mixture of inactive trypsin with a relatively small percentage of trypsin-enterokinase. A complete separation is necessary for certain specificity distinctions. An effective separation is obtained by

[1] The same, H. **149**, 221 and particularly p. 226 (1925).

[2] R. Willstätter, E. Waldschmidt-Leitz and A. R. F. Hesse, H. **126**, 143 and particularly p. 162 (1922–23).

[3] H. **161**, 191 (1926).

treating the glycerine extract with alumina gel at about *p*H 3.8. The resulting solution is neutralized with ammonia and again treated with alumina, the trypsin being adsorbed quantitatively at the neutral reaction. By elution with a glycerine-containing solution of casein the trypsin-enterokinase is obtained in its entirety; the inactivated trypsin remaining on the adsorbate may be obtained by elution with ammonia.[1]

The process thus described involves high losses but has been improved by a process of selective adsorption on casein rather than by selective elution with casein.[2] The modified procedure depends on the specific adsorption of trypsin-kinase on a precipitate of casein formed in the enzyme solution itself. This process is much more effective than adsorption by some insoluble substance as fibrin. Such specific adsorptions of enzymes by their substrate have been noted previously, as in the case of amylase by Ambard.[3]

This selective adsorption of trypsin-enterokinase by the substrate is considered as supporting the assumption that trypsin acts by the formation of an intermediate enzyme-substrate compound. The facilitating action of enterokinase, thus observed, suggests that proteases act as other enzymes by the intermediary formation of enzyme-substrate compounds.

Specificity of Trypsin.—The numerous statements in the earlier literature regarding the specific actions of pancreas trypsin have mostly proved untenable. In particular that assumption has proved fallacious which regarded as the specific substrate of trypsin only those high molecular proteins, for whose hydrolysis an activation of the enzyme by enterokinase was necessary. It depended on the prevailing view regarding the mode of action of kinase, i.e., that of an enzymic formation of trypsin from its zymogen. Thus, new procedures for differentiating the special substrates of pancreas trypsin and pancreas erepsin have been made possible by the positive establishment of the nature of this activation process; formerly all observed actions of non-activated pancreas preparates were ascribed to the action of erepsin. The separation of both these enzymes by the process of fractional adsorption by alumina, as carried out by Wald-

[1] E. Waldschmidt-Leitz, A. Schäffner and W. Grassmann, H. **156**, 86 (1926).

[2] E. Waldschmidt-Leitz and Kaj Linderstrøm-Lang, H. **166**, 241 (1927).

[3] Soc. Biol. **83**, 1458; **84**, 230 (1921).

schmidt-Leitz and Harteneck has likewise opened the way to a definite establishment of their specific actions and also to the significance of the auxiliary substance, enterokinase, for the specificity of the tryptic enzymes.

It has been found that a strict discrimination can be made between the special actions of trypsin and erepsin. Hydrolysis by trypsin is observed in all the genuine proteins examined, as also in the higher proteolytic degradation products, e.g., those formed by pepsin digestion, even though it is accomplished in some cases with widely divergent velocities. Ordinary peptides, on the other hand, such as di- or tri-peptides, are not attacked by trypsin—the ereptic enzyme is specific for their cleavage. Then too it has been shown that the influence of activation by enterokinase is essential for the hydrolysis of only the higher molecular proteins, and that on the contrary simply constituted substrates, e.g., certain basic proteins such as the protamines or products of peptic decomposition, are rapidly broken down even by the non-activated enzyme. In these cases the presence of the enterokinase is seen only in a strengthening and an extension of the hydrolysis. Meanwhile it has been shown in recent researches [1] that the attack of the activated and the non-activated trypsin relate to specific structures; their manner of action likewise is sharply differentiated. Table XXX illustrates the comparative

TABLE XXX

Specificity of Trypsin and Erepsin

(Signs indicate: − = not detectable; + = positive, ++ = increased cleavage)

Substrate	Trypsin	Trypsin Enterokinase	Erepsin
Peptide	−	−	+
Peptone from egg albumin	+	++	−
Protamine	+	++	−
Histone	−	+	−
Casein	−	+	−
Fibrin	−	+	−
Gelatin	−	+	−
Gliadin	−	+	−
Zein	−	+	−

[1] E. Waldschmidt-Leitz, A. Schäffner and W. Grassmann, H. **156**, 86 (1926); E. Waldschmidt-Leitz and E. Simons, H. **156**, 99 (1926).

specificity of pancreas trypsin and pancreas erepsin in qualitative form.

The special mode of action of trypsin and the exact nature of the chemical structures which decide its attack are still not clearly recognizable. But its action is expressed, as various researches have shown, in the cleavage of acid amide linkages and particularly in the formation of appreciable quantities of free amino acids. In this last relation, which seems to be essential, it differs, at least in a quantitative sense, from that of the peptic enzyme. The peptic enzyme, as stated before, forms only peptides and no amino acids.

c. Thrombin

It is doubtful whether we are justified in regarding the phenomenon of blood coagulation as a proteolytic or even as an enzymic process. An effort has also been made to explain it from a purely colloid chemical viewpoint. Within the compass of this book it may suffice to give a brief description of the coagulation process and the conditions which control the process and to omit more detailed study of its reaction mechanism. The similarity of this phenomenon to that of the lab coagulation of casein, which may be based not only upon an empirical analogy, but also upon an internal analogy, prompts us to a careful consideration of its enzymic principles. In both these cases, analytical investigations have not yet been sufficiently extensive to permit any conclusive decisions.

The phenomenon of blood coagulation depends upon the transformation of a protein, fibrinogen, dissolved in the blood serum, into insoluble fibrin. It was first attributed by Schmidt [1] to the action of the enzyme, thrombin, which is supposed to be given off into the blood upon the decomposition of cell elements, e.g., the red blood corpuscles, and this takes place in the form of an inactive pre-stage, which is next transformed into the active coagulating enzyme by the addition of lime salts. The significance of lime salts, which was first pointed out by Arthus and Pagès,[2] does not consist, according to Hammarsten,[3] as in the case with

[1] Pflüg. Arch. **6**, 413 (1872); **11**, 291, 515 (1875); **13**, 93 (1876).
[2] Arch. de Physiol. **2**, 739 (1890).
[3] H. **22**, 333 (1896–97) **28**, 98 (1899).

lab action, in its reaction with the transformed protein, the fibrin, but in its action upon the incomplete enzymes. From the activation of the latter, the presence of a special activator, thrombokinase, contained in the tissue juices, is necessary, according to Fuld [1] and Morawitz.[2] These observations recall the older ideas as to the activation of trypsin by calcium salts, whose action, as has been shown, depends merely upon an alteration of the reaction of the enzyme solutions. With regard to the individual views as to the mechanisms involved and the conditions affecting the coagulation of blood, reference might be made to the exhaustive special literature on this subject.[3]

d. Proteases of Higher Plants

Papain.—Papain or papayotin is found in the fruit or milky juice of the melon-tree (*carica papaya*) (better known as the papaw or papaya), whose protein digestive action has been known for a long time. The enzyme was first closely studied by Wurtz and Bouchout,[4] and very thoroughly by the botanist Vines,[5] and compared with plant proteases. The information gained from these works has been essentially extended and corrected by newer researches, in which attention has been called to the significance of a special activation of the enzyme by means of hydrocyanic acid.

This activation, first recognized as such by Mendel and Blood [6] and by Frankel,[7] has been extensively investigated by Willstätter and Grassmann [8] with reference to its mechanism and its significance for the specificity of the enzyme. It has been shown that the action of hydrocyanic acid on papain is very similar to the reaction between trypsin and enterokinase. The combining

[1] Zbl. f. Physiol. **17**, 529 (1903); E. Fuld and K. Spiro, Hofm. Beitr. **5**, 171 (1904).

[2] Hofm. Beitr. **4**, 381; **5**, 171 (1904); D. Arch. klin Med. **79**, 1 (1904).

[3] See P. Morawitz, Die Blutgerinnung. Handb. d. Biochem. **4**, 44, 2. Aufl. Jena, 1923.

[4] C. r. **89**, 425 (1897); **91**, 787 (1880); **93**, 1104 (1888).

[5] Annals of Botany **16**, 1 (1902); **17**, 237, 597 (1903); **18**, 289 (1904); **19** 149, 171 (1905); **20**, 113 (1906); **22**, 103 (1908); **23**, 1 (1909); **24**, 213 (1910).

[6] J. Biol. Chem. **8**, 177 (1910).

[7] J. Biol. Chem. **31**, 201 (1907).

[8] H. **138**, 184 (1924).

of the activator with the enzyme is a reaction conditioned by time; in the case of papain a period of one hour is required for the action of hydrocyanic acid to attain maximum activation. Furthermore, the papain-HCN compound is marked by a considerable capacity for dissociation; the activator can be withdrawn from the activated enzyme by a diminution of pressure. The activated and the non-activated enzyme, papain-HCN and papain, whose optimal actions on gelatin correspond to a *p*H of 5.0, and whose activity-*p*H curves coincide, differ in their specificity; they behave as two specific proteolytic enzymes. The activation of trypsin by enterokinase results in an increase of the substrates susceptible to the enzyme, the increase being particularly in the direction of the genuine proteins. In the case of the plant enzyme, its action is extended by the action of the co-enzyme, hydrocyanic acid, but conversely to trypsin, the extension is in the direction of the lower protein degradation products, the peptones and also the polypeptides. Thus peptones, whether obtained by peptic or by papain digestion, are not attacked by papain alone, but are split in a progressive and extensive reaction by the combination of papain and hydrocyanic acid.

The activator appears to be indispensable also for the hydrolysis of tri-peptides, while simple di-peptides are not attacked by papain-hydrocyanic acid, just as they are not attacked by trypsin. The rather unusual relations which appear to exist between the specificity of trypsin and that of papain, and also the influence of the special activators upon these are shown qualitatively in Table XXXI.

TABLE XXXI

SPECIFICITY OF PAPAIN AND TRYPSIN

(Signs indicate: − = not detectable, + = positive, ++ = increased hydrolysis)

Enzyme	Gelatine	Substrate Peptone	Dipeptide
Papain	+	−	−
Papain + Hydrocyanic Acid	++	++	−
Trypsin + Enterokinase	+	++	−
Trypsin	−	+	−

This table shows the justification for contrasting papain with trypsin-enterokinase on the one hand, and of papain-hydrocyanic acid with trypsin on the other, even though further research may indicate that an equivalence of the two enzyme types with respect to their specific action is not to be assumed. Further, the extension of the region of specific action in proteolysis, which is induced by the activator and the direction of which is found to be opposite for the two enzymes, is of different extent also in the case of the two enzymes. This may even arise from the different position observed with respect to a tri-peptide, which is markedly broken down by papain-HCN, but not by trypsin.

The preparative behavior of papain from the dried milky sap of *carica papaya* has also been tested by Willstätter and Grassmann; however, the increase in enzymic concentration was inconsiderable, being about five times that of the source material. The adsorption behavior of the enzyme is rather remarkable. (Before the experiments it was subjected to a pre-purification by self-digestion of the milky sap and precipitation by alcohol.) Although the adsorption of papain on kaolin appears to be only slightly affected by the reaction, its adsorption by alumina is found to be by far the most favorable in alkaline reaction and the enzyme was extracted again from the adsorbent by the action of the dilute acid. The basic properties apparently possessed by the enzyme or its association products are weakened by the addition of alcohol, which facilitates the adsorption on alumina—an action that may be compared with the development of the acid function observed in amino acids and peptides.[1]

Bromelin.—The protease of *ananas* (pineapple), which is known in the literature as bromelin, is of the same nature as papain, the only distinction being that the protease of *ananas* acts weakly on peptone.[2] This difference might be explained by possible association with a natural activator.

Due to lack of consideration of the *p*H influence, Vines [3] has reported that bromelin is inhibited by HCN. In the more recent studies, however, it is shown positively that bromelin is activated by HCN in the same way as papain. Both papain and bromelin

[1] Compare R. Willstätter and E. Waldschmidt-Leitz, B. **54**, 2988 (1921).
[2] R. Willstätter, W. Grassmann and O. Ambros, H. **151**, 286 (1926).
[3] Annals of Botany **17**, 597 (1903); **19**, 149 (1905).

are also activated by H_2S, the activation of papain by H_2S having been demonstrated by Mendel and Blood.[1]

Protease of Pumpkin.—On the other hand, the protease of pumpkin appears to be distinctly different from the above two proteases. Its activity is inhibited both by HCN and H_2S. The optimum activity of pumpkin protease on gelatin occurs in a weakly alkaline region, that of papain at *p*H 5.0. Pumpkin protease is easily adsorbed by alumina in acid solution, whereas papain is adsorbed only from a weakly ammoniacal solution. Pumpkin protease acts very poorly on gelatin but with relatively greater velocity on fibrin, wherein it is differentiated from papain-HCN. The relative sensitiveness toward alcohol is another point of distinction between the two proteases.

Hydrogen-Ion Dependence.—The activity of the two enzymes papain and bromelin, is not in conflict with the electro-chemical theory proposed by Northrop.[2] According to this assumption, as mentioned before, pepsin, which reacts only with protein kations and trypsin, which reacts only with anions, should exhibit activity-*p*H curves which coincide with the dissociation curves of the protein substrate. Willstätter, Grassmann and Ambros [3] find that carica protease, with or without HCN, acts most strongly on gelatin at approximately the isoelectric point of the substrate, namely at *p*H = 5.0. Optimum activity of the same protease on albumin peptone takes place at *p*H 5.0 to 5.2, which is near the isoelectric point of the substrate (4.8). With blood fibrin, cyanide activated papain exhibits a sharp optimum at *p*H 7.1 to 7.3 (isoelectric point 7.2). The cleavage of the strongly basic clupein also appears to be in agreement, since Rogozinsky [4] has reported its optimal cleavage to be in an alkaline reaction. Bromelin, similarly, exhibits an optimum with gelatin between *p*H 4.5 and 5.0 and with albumin peptone at *p*H 5.0.

The behavior of pumpkin protease, however, is radically divergent. With peptone, the activity-*p*H curve exhibits a sharp optimum at *p*H 6.3, while gelatin is maximally hydrolysed in a weakly alkaline region. Only for the hydrolysis of the insoluble substrate, fibrin, does pumpkin protease exhibit an optimal region in agreement with that of papain (*p*H = 7.2 to 7.4).

[1] J. Biol. Chem. **8**, 177 (1910).
[2] J. Gen. Physiol. **5**, 263 (1922–23).
[3] H. **151**, 307 (1926).
[4] H. **79**, 398 (1912).

Willstätter, Grassmann and Ambros entertain the possibility that associated materials, either activating or inhibiting, can thus influence the characteristics of this enzyme, but they consider it more probable that the protease of pumpkin is a special type of plant protease, distinctly different from papain and bromelin.

Associated Ereptase.—The ereptic enzyme, which is limited exclusively to the hydrolysis of peptides, does not appear to be associated with the proteases in the dried milk juice of the papaw, although a weakly active ereptic compound occurs in association with the pumpkin protease. Dried preparates of bromelin from the pineapple appear to be inactive against peptides, although a small degree of ereptic activity has been noted in the expressed juice from fresh pineapples.[1]

Malt Proteases.—Among those proteases of the higher plants most worthy of mention are the enzymes of resting and germinating plants which effect the mobilization of the vegetable reserve-proteins. In the discussion of ricinus lipase, we have already spoken of their special significance for the inauguration of fat cleavage in oleaginous seeds. Those best studied appear to be the proteases of malt, in which, according to the researches of Adler [2] and of Lundin [3] the presence of two different proteolytic enzymes must be assumed, a protease and a peptidase, whose difference is based on their *p*H-dependence. It has not yet been investigated whether, as is probable, the protein-cleaving enzyme of malt is related to papain with respect to its specificity. Also the much studied formation of proteolytic enzymes in germinating seeds, in which a special conjunctive action of oxygen has been assumed, requires verification with improved methods, which must exclude above all the inadequately controlled influence of acidity.

e. Yeast Proteases

The observation that in the autolysis of yeast the proteins contained therein are broken down into soluble products is already old; it was first attributed by Salkowski [4] to enzymic action. Hahn and Geret [5] have obtained evidence that the press-juice of

[1] R. Willstätter, W. Grassman and O. Ambros, H. **152**, 160 (1926).
[2] Z. ges. Brauw. **38**, H. 17-20 (1915).
[3] Bio. Z. **131**, 193 (1922).
[4] H. **13**, 506 (1889).
[5] Z. Biol. **40**, 117 (1900).

yeast is capable of hydrolyzing genuine proteins and that in this reaction large quantities of amino acids are formed, whereas the decomposition stage, peptone, was shown to be absent. They compared the proteolytic degradation by yeast enzyme with that of trypsin and termed the enzyme endo-tryptase, although they had already made the observation that the optimal action of the enzyme, differing from that of trypsin, corresponded to a feebly acid reaction, as likewise it corresponded to the most favorable condition for the autolytic process. The knowledge of yeast proteases was next essentially advanced by a further investigation of much wider scope carried out by Dernby.[1] The optimal region for autolysis of yeast protein was found by Dernby at a *p*H of about 6.2, while its limits were determined as *p*H = 4 or 7. According to Dernby, three different proteolytic enzymes take part in this process, whose differentiation is expressed on the basis of their differing *p*H-dependence. Thus the action of a protease optimal at *p*H 4.5, differs from that of a tryptase and a peptidase with action-optima at *p*H 7.0 or 7.8; the first two enzymes are capable of hydrolysing genuine proteins or peptones, the third is specific for simple peptides. From the mutual interplay of the actions of these three enzymes, the picture of the autolysis is given, whose *p*H-dependence, as shown in Fig. 11, is derived from the activity-*p*H curves of the enzymes taking part.

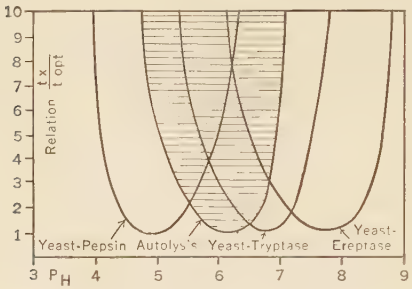


FIG. 11.—Activity-*p*H curves of yeast proteases. (*According to Dernby.*)

These conclusions were not based on a separation of the enzymes participating, but were based merely on the different *p*H optima observed. More extensive investigations by Willstätter and co-workers [2] have led to a characterization of three proteolytic enzymes in yeast which is considerably different from that reported by Dernby. It has been shown by preparative methods

[1] Bio. Z. **81**, 107 (1917).

[2] R. Willstätter and W. Grassmann, H. **153**, 250 (1926); W. Grassmann and W. Haag, H. **167**, 188 (1927); W. Grassmann and H. Dyckerhoff, H. **175**, 18 (1928); W. Grassmann and H. Dyckerhoff, B. **61**, 656 (1928).

that yeast contains three enzymes, one of which acts on proteins and peptones, one on polypeptides, and one on dipeptides. The enzyme acting on proteins exhibits an optimum at *p*H 5.0 and those acting on peptides at *p*H 7.0 to 7.8. The protein-attacking enzyme of yeast does not act to any appreciable degree on native egg albumin; on the other hand, its activity is very pronounced in the case of denatured egg albumin, denatured serum albumin, fibrin, gelatin, casein and certain peptones. The preparation of a polypeptidase-free protease and a protease-free polypeptidase as carried out in the brief autolysis of yeast in alkaline reaction, is not very satisfactory from the standpoint of enzyme concentration but nevertheless appears to be sufficiently effective for the separation of these two enzymes.[1]

The point of demarcation between the polypeptide splitting enzymes and the protein splitting enzymes does not seem to be conditioned by the length of the polypeptide chain, as will be shown later to be the case with the di- and polypeptidases of yeast. Rather, the specificity distinction appears to be due to different types of structural units in the protein molecule.

2. PEPTIDASES

The peptide-splitting enzymes of the plant and animal kingdoms, which are radically different from the ordinary proteases, are to be regarded as the simplest examples of proteolytic enzymes. The exact demarcation of their special actions from that of the usual proteases, however, has been carried out only very recently. The facility with which an intimate study can be made of the comparatively simple reactions catalyzed by these enzymes seems to render their investigation peculiarly fitted to contribute to an understanding of the mechanism of proteolytic processes; it is to be expected that they will have a significant role in the recognition and solution of structural problems in protein chemistry.

Intestinal Erepsin.—An important and carefully studied peptidase is the erepsin of the intestinal mucous membrane, discovered by Cohnheim[2] in 1901. Its discovery was based on the observation that certain protein degradation products were broken down by extracts of intestinal mucous membrane to the point of

[1] W. Grassmann and H. Dyckerhoff, B. **61**, 656 (1928).

[2] H. **33**, 451 (1901).

disappearance of the biuret reaction—to amino acids. This degradation was applicable both to the proteins pre-digested with pepsin and the protein degradation products incapable of further degradation by trypsin, the trypsin-peptones first described by Kühne.[1] The statements in the earlier literature with regard to the special modes of action of the intestinal erepsin, according to which there were included among its specific substrates most of the synthetic polypeptides as well as the peptones of peptic and tryptic disgestion, and likewise protamine, histone and casein, have been shown to be entirely unreliable by a recent investigation by Waldschmidt-Leitz and Schäffner.[2]

Along the same lines also the distinction made in the literature between the specific substrates of the pancreas trypsin and the pancreas erepsin has been contradicted and corrected by Waldschmidt-Leitz and Harteneck with the separation of the two enzymes and the recognition of their separate modes of action. It has been found thereby that the enzymically integral erepsin from the intestine as well as from the pancreas is specific for the hydrolysis of ordinary peptides and for these alone. Neither the peptone of pepsin digestion, nor protamine, nor histone, nor any other proteins whatever are attacked by the pure enzyme. The assertions in the literature regarding the susceptibility of such substrates must be attributed rather to an admixture with trypsin, the separation of which can be accomplished by fractional adsorption with alumina. The same specificity, the restriction of their action to the hydrolysis of simple, natural or synthetic polypeptides, is to be assumed for the plant peptidases, e.g., for the peptidases of yeast, which have been obtained by Willstätter and Grassmann [3] in enzymically integral solution.

Identity of Pancreas and Intestinal Erepsin.—The qualitative and quantitative comparison of the specificity of intestinal and pancreatin erepsin, which Waldschmidt-Leitz and Schäffner have tested, has led to the establishment of the fact that there is no longer any reason to believe that the two enzymes are different, as was assumed on the basis of earlier reports by Fischer and Abderhalden.[4] It may be added that both the adsorption behavior

[1] Virch. Arch. **39**, 130 (1867); Z. Biol. **22**, 450; **28**, 571; **29**, 1, 308.
[2] H. **151**, 31 (1925–26).
[3] H. **153**, 250 (1926).
[4] H. **46**, 52 (1905).

of the enzymes with respect to alumina and the dependence of their activity upon the H-number have been found to be in agreement; they are, accordingly, to be considered as identical. This conclusion leads, according to Waldschmidt-Leitz and Harteneck,[1] to a more extensive physiological inquiry; it must be assumed that the primary formation of even the intestinal erepsin itself is accomplished in the pancreas, and that its excretion by the intestinal mucous membrane is preceded by a secondary accumulation in the gland cells of the latter.

From the researches of Fischer and Abderhalden, furthermore, the significant fact first became known that the enzymic hydrolysis of the synthetic polypeptides proceeds by an asymmetric course. The observations of the newer literature also agree that the peptidases are capable of splitting only those peptides built up from the naturally occurring optical antipodes of the amino acids; and in the racemates of the synthetic polypeptides only one is subject to hydrolysis, namely, the one corresponding to the naturally occurring steric isomer.

Hydrogen-Ion Dependence of Erepsin.—The *p*H optimum of intestinal erepsin has been determined by Rona and Arnheim [2] as well as by Dernby [3] to be 7.8. The position of the optimum, which, according to Dernby, is found to be identical with the intestinal and the yeast enzyme for the cleavage of glycyl-glycine, varies, according to Abderhalden and Fodor,[4] with the plant enzyme within certain limits, between $pH = 6.2$ and $pH = 8.5$, depending on the nature of the peptide employed. Waldschmidt-Leitz and Schäffner,[5] however, were unable to verify this observation for animal erepsin.

Kinetics.—The reaction course, also, of peptide hydrolysis appears to differ with the animal and plant enzymes. Whereas the researches of Euler,[6] as well as those of Dernby with respect to the hydrolysis of glycyl-glycine by intestinal erepsin led to the establishment of mono-molecular reaction equations, Abderhalden and Fodor hold that the time-course of peptide-cleavage by a plant enzyme, from the press-juice of yeast, depends in large measure on the H-number. Their experiments gave with $pH = 6.3$ a direct proportionality between transformation and time, while

[1] H. **149**, 221 (1925).
[2] Bio. Z. **57**, 84 (1913).
[3] Bio. Z. **81**, 107 (1916–17).
[4] Fermentf. **1**, 533 (1916).
[5] H. **151**, 31 (1925–26).
[6] H. **51**, 213 (1907).

with pH = 7.4 the validity of the equation of the first magnitude was observed, but with pH = 8.4, that of the Schütz law was observed, under conditions wherein there was no question of enzyme destruction. This behavior recalls the phenomena of varying kinetics observed in the case of the cane-sugar splitting enzyme of yeast, the dependence of which upon acidity has been described by Willstätter, Graser and Kuhn.[1] This influence of the H-number, however, does not apply, according to Waldschmidt-Leitz and Schäffner, to the action of animal erepsin, which corresponds to the mono-molecular reaction equation; it would seem, either that the reaction mechanism of plant and of animal peptidase is different, or else that the affinity of the yeast erepsin to its substrates is strongly affected by concomitant substances.

The scale proposed by Waldschmidt-Leitz and Schäffner[2] for measuring the amount and concentration of erepsin is based upon the monomolecular course of peptide hydrolysis and upon the observed proportionality between the enzyme quantity and the reaction velocity. As "erepsin-unit (Er. E)" the 1000-fold amount of that enzyme quantity is chosen which, under the conditions of the determination, gives the reaction constant of 0.001. The "erepsin-value (Er. W)," as a measure of the enzymic concentration, is given by the number of erepsin-units in 1 cg. of the preparates.

Preparative Purification.—The preparative purification of erepsin, which has been attempted by Waldschmidt-Leitz and Schäffner with the enzyme of the intestinal mucous membrane, is affected in its result by the extremely low stability of the enzyme. Only the glycerine-containing media, which have a stabilizing effect, are suited for extracting the enzyme from the mucous membrane. The adsorption of the enzyme on alumina is accomplished readily in acid solution, e.g., in extracts previously purified by precipitation with acetic acid. This adsorption has an analytical importance of the first order, since it effects the separation of the enzyme from the tryptic impurities always associated with it; these impurities remain behind in the mother-liquor of the

[1] H. **123**, 1 and particularly p. 63 (1922).

[2] Same as before; see also E. Waldeschmidt-Leitz and A. Harteneck, H. **147**, 286 and particularly p. 295 (1925).

adsorption. This method has decisively advanced our knowledge as to the specific modes of action of erepsin.

Specificity of Peptidases.—The new methods by which ereptases and trypsin may be obtained in enzymically integral form have permitted sharp demarcations as to their region of specificity. To begin with, it has been shown that two distinctly differentiated peptidases occur in yeast autolysates. Grassmann and Haag [1] have separated a dipeptidase from a polypeptidase by selective adsorption and elution in a medium of alumina gel. The yeast dipeptidase hydrolyses all dipeptides with considerable rapidity but is entirely inactive against tri-, tetra- or higher peptides. The nature of the amino acid structural units in the dipeptides appears to be without significance. Recent experiments show that even the dipeptides, serine, tyrosine and glutamic acid, are split by dipeptidases.[2] Conversely, the yeast polypeptidases are totally inactive against dipeptides, but rapidly hydrolyse polypeptides containing three or more amino acid residues. The differentiation between polypeptidases and those proteases which hydrolyse casein is not clear, but is probably conditioned by the nature rather than the number of amino acid residues.

According to Waldschmidt-Leitz, Grassmann and Schlätter,[3] animal peptidases in general hydrolyse all simple peptide chains, apparently regardless of length. The distinction between animal erepsin and trypsin is not conditioned merely by the length of the peptide chain but by the nature of the structural units. Introduction of glycine residues up to an octadecapeptide still yields a substrate hydrolyzed only by the erepsin. This differentiation of yeast ereptase and animal ereptase is in contradiction to the earlier assumption of Dernby as to their identity.

However, upon introduction of tyrosine into the tetrapeptide, leucyl triglycine, forming the pentapeptide, leucyl triglycyl tyrosine, a qualitative specificity variation is produced. The resulting peptide is now converted by trypsin but not by erepsin. That the length of the peptide chain is of importance for this specificity variation is indicated by the comparison of the enzymic susceptibility of leucyl triglycyl tyrosine, on the one hand, and of

[1] H. **167**, 188 (1927).
[2] W. Grassmann and H. Dyckerhoff, B. **61**, 656 (1928).
[3] B. **60**, 1906 (1927).

simple glycyl tyrosine, on the other, the latter being specific for the ereptase.

TABLE XXXII

SPECIFICITY OF PANCREAS TRYPSIN AND INTESTINAL EREPSIN

(Signs indicate: − = not detectable, + = positive hydrolysis)

Peptide		Trypsin (Activated)	Erepsin
Dipeptides	Glycyl glycine	−	+
	Leucyl glycine	−	+
	Glycyl tyrosine	−	+
Triglycine		−	+
Tetraglycine		−	+
Leucyl triglycine		−	+
Pentaglycine		−	+
Hexaglycine		−	+
Leucyl heptaglycine		−	+
Leucyl trigylcyl tyrosine		+	−

This finding has been further confirmed and amplified by Waldschmidt-Leitz and co-workers.[1] Examination of a number of polypeptides and peptide derivatives has shown that intestinal erepsin attacks peptide chains containing a large number of amino acids but is restricted to some of the substituted amino acids.

A still more intimate definition of the region of specificity has been pointed out by Waldschmidt-Leitz and Klein,[2] who show that a specific substrate for erepsin in converted into a trypsin substrate upon introduction of acyl groups. The rule was found to hold generally for all compounds examined as shown by Table XXXIII.

[1] E. Waldschmidt-Leitz, A. Schäffner, H. Schlätter and W. Klein, B. **61**, 299 (1928).

[2] B. **61**, 640 (1928).

TABLE XXXIII

SPECIFIC SUSCEPTIBILITY OF SUBSTITUTED PEPTIDES

(Signs indicate: − = not detectable, + = positive hydrolysis, 0 = not tested)

Substrate	Intestinal Erepsin	Trypsin-Kinase
β-Naphthalinsulfonyl glycyl tyrosine	−	+
Glycyl glycine carbonic acid	−	0
Carboethoxyl glycyl leucine	−	+
Carboethoxyl pentaglycine	−	0
Acetyl glycyl glycine	−	+
Acetyl (phenyl alanyl) alanine	−	+
Benzoyl diglycine	−	+
Benzoyl triglycine	−	+
Benzoyl tetraglycine	−	0
Benzoyl pentaglycine	−	−
Phthalyl diglycine	0	+
Glycyl leucine amide	+	−
Tetraglycine amide	+	−

3. MECHANISM OF PROTEIN AND PEPTIDE DEGRADATIONS

Peptidase Action.—The preparation of integrally pure enzymes, acting on these relatively simple substrates, has provided a more profound insight into the mechanism of substrate cleavage. For example, Grassmann and Dyckerhoff [1] have characterized the stepwise mode of attack which takes place in the degradation of tri- and tetrapeptides. It has been known that the action of polypeptidase on tripeptides comes to a sharp standstill after decomposition into one mole of amino acid and a mole of dipeptide. However, it remained for these investigators to settle decisively the question as to which of the end amino acids in the chain is liberated.

Abderhalden and co-workers [2] twenty years ago carried out a careful series of investigations in relation to this problem. The course of the hydrolysis of active tri- and tetrapeptides was followed

[1] H. **175**, 18 (1928).

[2] H. **54**, 363 (1907–08); **55**, 416 (1908); **57**, 342 (1908); **62**, 145 (1909); **66**, 277 (1910); **81**, 1 (1912).

by means of the optical rotatory power. The rotatory powers of the fission products are sufficiently distinct in certain cases to give an indication of the course of the reaction, and the procedure in general is particularly convenient. However, as Abderhalden himself has shown, the method is not consistently reliable. In the light of modern enzyme chemistry, a number of objections can be raised against this series of investigations. Most important, the H-ion concentration was not observed, and the enzymes were insufficiently characterized.

Using integrally pure enzyme preparates, Grassmann and Dyckerhoff have quantitatively separated and identified the fission products. *In all cases the end amino acid carrying the free amino group is the one split off from the polypeptide chain.* In the case of leucyl glycyl glycine, leucine and glycyl glycine were separated in almost quantitative yield. (The leucine is separated from the fission products by means of its difficultly soluble copper salt and the glycyl glycine as the ester chlorohydrate.) In the case of glycyl glycyl-l-leucine, the peptide is shown to be split into glycocoll and glycyl leucine. The peptide linkages in the remaining di- or tripeptide appear to be wholly resistant against further action of the enzyme in the reaction mixture. It is not entirely clear as to why the reaction comes to a complete standstill after liberation of one carboxyl group.

This strictly specific action of the enzyme stands in contrast to the unspecific course of alkali hydrolysis. Thus, Abderhalden and Brockmann [1] recently showed that in the incomplete alkali hydrolysis of l-leucyl-glycyl-d-alanine, both leucyl glycine and glycyl alanine can be demonstrated.

The fact that a free amino group in neighboring proximity to the peptide linkage is necessary for the action of the enzyme, is supported by the previous findings as to the resistance of peptide derivatives with substituted amino groups. Thus it has been shown that yeast polypeptidase splits neither benzoyl diglycyl glycine nor β-naphthalinsulfo-glycyl-tyrosine. These, however, are substrates for the action of pancreas trypsin, as has been shown by Waldschmidt-Leitz and co-workers.

A particularly significant correlation at this point is the fact that the activity-*p*H curve for erepsin with glycyl-glycine corresponds very closely to the condensation of glucose with glycyl

[1] H. **170**, 147 (1927).

glycine. This was recently demonstrated by Waldschmidt-Leitz and Rauchalles [1] and has been described in the first portion of this volume in relation to the possible aldehyde nature of enzymes. It is a striking phenomenon that the glucose-peptide condensation should reach a maximum at exactly the H-ion concentration (*p*H 8.0) as the maximum activity of erepsin on the same peptide. In view of the several facts given above, some type of combination between the enzyme and the free amino grouping would seem to be a very real probability.

Grassmann and Dyckerhoff have further pointed out that for the action of yeast dipeptidase it is necessary that there be a free carboxyl grouping in neighboring proximity to the normal peptide linkage and that the carboxyl must be in the usual characteristic dipeptide position. Accordingly the carboxyl group of a dipeptide can be so varied as to render the compound resistant against the action of dipeptidases. The following modifications are not attacked by dipeptidases: esters of dipeptides; amides of dipeptides, among which may be counted the higher polypeptides; decarboxylated dipeptides; and finally dipeptides of the type of β-amino butyric acid. The latter contain carboxyl groups, but not, however, in the usual neighboring position to the peptide linkage.

Esters of dipeptides and dipeptide amides are split very easily and rapidly by yeast polypeptidases. In other words it can be concluded that the neighboring position of a free carboxyl group hinders the attack of a polypeptidase.

The conclusions thus developed for the peptidases may be arranged in the following simple scheme:

	Neighboring of a Free	
	NH_2 Group	COOH Group
Yeast Dipeptidase	Necessary	Necessary
Yeast Polypeptidase	Necessary	Hinders
Intestinal Erepsin	Necessary	Unnecessary and immaterial

[1] B. **61**, 645 (1928).

Protease Action.—The application of integrally pure enzymes to the fractional hydrolysis of true proteins, histones and protamines has further permitted an insight as to the mechanism of their degradation. In the example of a protamine, the clupein from herring milk, Waldschmidt-Leitz, Schäffner and Grassmann [1] have demonstrated that the hydrolytic capacities of the various proteases participating in the degradation stand in whole-number relations to one another and further that the total process of its hydrolytic splitting consists in the rupture of peptide linkages. Also the fractional hydrolysis of a higher molecular protein, casein, leads to similar results with respect to the quantitative participation of the individual proteases, even though the degradation in not complete.[2]

The use of enzymic analysis as a means for the characterization and classification of proteins has been recently suggested. Heretofore proteins have been differentiated principally according to their occurrence or according to their solubility behavior. With the former type of classification in mind, Waldschmidt-Leitz and Kollmann [3] have studied the fractional degradation of four protamines: clupein, salmine, scombrine and sturine. Many of the observations of the older literature [4] concerning the enzymic splittability of protamines were not carried out with definite and individual enzyme materials. The experiments by Waldschmidt-Leitz and Kollmann involved the use of enzymically integral trypsin, trypsin-kinase, papain-HCN, papain and erepsin, the latter two being inactive against the substrates mentioned. All the first three enzymes are active against each of the substrates except sturine. Quantitative comparisons, however, give greater divergences. Clupein and salmine are approximately the same, whereas scombrine and sturine are hydrolyzed to a considerably less extent. For clupein the fraction of carboxyls liberated by trypsin amounts to one-fifth of the total enzymically liberated groups; for scombrin it amounts to only about one-ninth; and for sturgeon protein it is practically negligible.

[1] H. **156**, 68 (1926).

[2] E. Waldschmidt-Leitz and Simons, H. **156**, 99 (1926).

[3] H. **166**, 262 (1927).

[4] Compare A. Kossel and A. Matthews H. **25**, 190 (1898); O. Cohnheim, H. **35**, 134 (1902); A. Kossel and H. D. Dakin, H. **41**, 321 (1904); M. Takemura, H. **63**, 201 (1909); F. Rogozinski, H. **79**, 398 (1912).

Such differences are to be sought for in the structural individuality, either in a varying molecular magnitude or in a distribution of definite structural units. From work by Kossel on the relative structures it would appear that the tryptic capacity thus described corresponds to the proline content of the protamines.

The hydrolysis of histones as carried out by Waldschmidt-Leitz and Künstner [1] can be considered as also having a bearing on the structural characteristics of these particular substances. Histones belong to the simpler groups of protein substances and are related to the protamines, their predominantly basic properties being mutually characteristic. They are differentiated from the protamines by a more complicated composition which includes a greater number of amino acids. Histones therefore may be considered as the simplest members of the high molecular protein groups.

Felix [2] supposed that, in contrast to the proteins, the guanidine group of the arginine residue as well as the ϵ-amino group of lysine are involved in the structure of this protein. It has been further suggested that the specific task of pepsin in the degradation of histones and also other proteins consists in the rupture of such acid amide linkages.

This last assumption is decisively contradicted by the fact that the mode of action of pepsin is not individual but consists merely in the rupture of ordinary peptide linkages, recognizable by the uniform increase of carboxyl and α-amino acid groups. Waldschmidt-Leitz and Künstner have shown that the function of pepsin in the fractional degradations of histone consists in the rupture of about one-tenth of the linkages involved in the complete hydrolysis, standing in simple relation to the capacity of other enzymes. Also it is shown that its action may be replaced by pancreatic enzymes and in degradations in which the enzymic hydrolysis is complete. That is, the degree of hydrolysis in such cases corresponds to that attainable by acid hydrolysis. Accordingly, it is considered that there is no basis for the assumption that the guanidine groups of arginine or the ϵ-amino groups of lysine are involved in the peptide linkages of histone.

The extension of such methods of attack gives promising evidence of providing a much more profound insight into the structural characteristics of the high molecular proteins.

[1] H. **171**, 290 (1927). [2] H. **120**, 94 (1922); **146**, 103 (1925); **165**, 103 (1927).

CLASSIFICATION OF THE PROTEASES

Many attempts have been made in the recent literature to advance a rational classification of the various proteolytic enzymes, according to their mode of action. Northrop [1] prefers, as already mentioned, a classification of the proteases according to the ionization state of the protein particles which enter into reaction with them. Dernby [2] distinguishes between primary and secondary proteases, corresponding to the types of pepsin and trypsin; and Oppenheimer [3] makes a distinction between pepsinases and eryptases. These distinctions, whose justification depends primarily upon the differences in optimal acidity relations observed for the enzyme actions, must be extended and, in a chemical sense, be rendered more profound, in accordance with the recent information obtained for the specific allocation of the proteases. As mentioned before, the hypotheses advanced particularly by Herzog [4] as well as by Abderhalden [5] as to the structure of proteins as polymeric substances, built up of units of simple basic substances, have led to a conception of the action of trypsin and papain as being primarily a phenomenon of disaggregation. They have also suggested that only a subordinate significance be attached to the observed differences of affinity which are based upon the structural constitution of their specific substrates.[6]

According to the views of the older literature, the hydrolysis of high molecular proteins is never complete in any of the possible combinations of pepsin, trypsin and erepsin, which contain only two components; and in the simplest cases, the degradation of protamines, only the pepsin can be dispensed with. These conceptions have been decisively invalidated by the experiments cited in the foregoing treatment.

It is further obvious that for a general representation of these substances their attack must be referred to specific substrates. Thus, the new information acquired with respect to the specificity

[1] Naturw. **11**, 713 (1923).
[2] Bio. Z. **133**, 432 (1922).
[3] Bio. Z. **136**, 140 (1923).
[4] Naturw. **11**, 172 (1923).
[5] Naturw. **12**, 716 (1924).
[6] See C. Oppenheimer, Die Fermente und ihre Wirkungen, 5. Auflage, Leipzig 1925, 2 Bd. p. 811 and 890.

of the animal proteases, in the researches of Willstätter and co-workers, has led to a differentiation of four types of proteolytic enzymes. To the *first group*, the peptidases, belongs erepsin, for which the specific substrates to be taken are simple peptides, e.g., di- or tri-peptides. A *second group* includes the enzymes of the type of non-activated trypsin, the modes of action of which have indeed been indicated but which cannot as yet be characterized with a strictly specific substrate. For the *third group*, activated trypsin, accordingly the system trypsin + enterokinase, can be taken as a characteristic member. For the *fourth group* pepsin may be taken; its specific actions cannot yet be described with certainty but can be differentiated from the other groups.

A classification of the various plant proteases obviously could not coincide entirely with such a classification for animal proteases. The plant proteases—yeast dipeptidase, yeast polypeptidase, yeast trypsin, papain, papain-HCN, bromelin, malt proteases, etc.—have definite points of specificity, which cannot yet be subordinated to any comprehensively general system of classification.

III. AMINOACYLASES

To the group of aminoacylases, which must be strictly differentiated in their specificity from the peptidases, there belong several important enzymes for which the hydrolysis of acylated amines or amino acids may serve as a common characteristic. Their individual members, however, may be distinguished from each other by special modes of action, e.g., the scission of acylamino acids, or the hydrolysis of urea and of guanidine derivatives.

The following treatment is limited essentially to the description of the three most important enzymes of this group—histozyme, arginase and urease.

1. HISTOZYME

Occurrence and Actions.—The ability of the kidney to synthesize and to split benzoylated amino acids, e.g., hippuric acid (benzoyl glycocoll), was ascribed by Schmiedeberg [1] to the action of a special enzyme, histozyme. This enzyme has been found also in the liver and in the muscle of mammals, and has been described especially by Neuberg [2] in the mold fungus *aspergillus oryzae*. According to Willstätter, Waldschmidt-Leitz and Waldschmidt-Graser [3] its presence is most considerable in the kidney of dogs and cats, whereas the enzyme content is greatly diminished. The action of histozyme is confined to the splitting off of the acid residue in acylated amino acids and peptides, e.g., the breaking down of benzoyl alanine, benzoyl-glycyl-glycine, phenaceturic acid and even glycocholic acid, whereas the true peptide linkage, e.g., in glycyl-glycine, remains unattacked. The optical specificity of the enzyme was first described by Smorodinzew [4]

[1] Virch. Arch. **14**, 288, 379 (1881).
[2] Bio. Z. **145**, 186; **147**, 370, 372 (1924).
[3] *Yet unpublished.*
[4] H. **124**, 123 (1922).

with the example of benzoyl leucine. Willstätter, Waldschmidt-Leitz and Waldschmidt-Graser found this optical specificity so pronounced that in almost all cases only the derivatives of the naturally occurring amino acid antipodes were broken down; this, further, was independent of the origin of the enzyme. Hoppert [1] accordingly has recommended the utilization of the enzyme for the resolution of racemic amino acids. The hydrolysis of hippuric acid by histozyme, according to Mutch,[2] follows a reaction equation of the first order; it progresses to an equilibrium corresponding to a cleavage of about 97 per cent. The fermentative synthesis of hippuric acid, however, has not yet been experimentally demonstrated with certainty.

Solubility.—The solubility behavior of the animal enzyme is very remarkable, varying considerably with the type of animal under investigation. Thus, the enzyme from the dog kidney is found to be completely soluble in aqueous media or glycerine, that of the swine kidney only partially soluble and that of the horse kidney almost entirely insoluble. This phenomenon is to be explained not by the assumption of absolutely different enzymic individuals, but by a differing tenacity in the linkage between the enzyme and the insoluble associated materials.

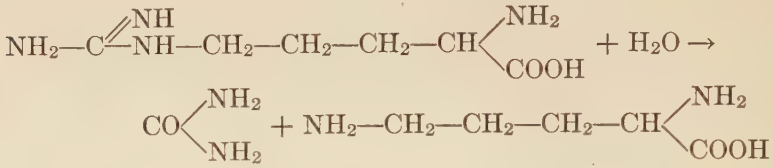
It has not yet been determined whether histozyme plays a part in the transformation of uric acid into allantoin. This transformation is constituted by a hydrolytic and an oxidative process and has hitherto been considered as due to a separate urilolytic enzyme in the kidney. This former theory appears all the more probable following the observations of Wiechowski.[3] According to these observations, uric acid is destroyed in the human kidney but no allantoin is formed, the phenomena being explained by the absence of a specific oxidizing enzyme in this case.

2. ARGINASE

Action.—Arginase, as an enzyme specifically allocated to arginine, was first discovered and described by Kossel and Dakin.[4] Its action consists in the decomposition of arginine, guanido-amino

[1] Bio. Z. **149**, 510 (1924).
[2] J. Physiol. **44**, 176 (1912).
[3] Hofm. Beitr. **9**, 246, 295 (1907).
[4] H. **41**, 321 (1904).

valeric acid, into diamino valeric acid or ornithine and into urea according to the equation



Occurrence and Relation to Sexual Activity.—Edlbacher [1] has devoted a series of researches to the occurrence and the quantitative determination of arginase. It has been found therein that the enzyme is present in the liver of mammals, but not, on the other hand, in that of birds; it is found in larger amounts in the kidney and testicles of all the animals examined, whereas it appears to be almost entirely lacking in the ovaries. From the fact that the arginase content in the organs of male animals always greatly exceeds that in organs of the female, the conclusion has been drawn that male individuals have a hydrolytic arginine transformation which is greatly augmented as compared with that of females and that the arginine metabolism appears to have a definite relation to sexuality. At the same time it has been shown that the formation of arginase undergoes a marked increase upon the arrival of sexual maturity. These relations between sexuality and arginase content become comprehensible when we recall that basic proteins, rich in arginine, predominate in the sperm, and that consequently the organism will require an increased amount of the arginine-transforming enzyme at the period of sexual maturity.

Arginase is widely distributed in plants also, according to A. Kiesel.[2]

Determination.—The use of formal titration according to the Sörensen method is recommended by Edlbacher [3] and Clementi [4] for the determination of arginase action—this permits a determination of the amino groups of ornithin which are set free; whereas the urea which is likewise formed does not react with for-

[1] S. Edlbacher and P. Bonem, H. **145**, 69 (1925); S. Edlbacher and H. Röthler, H. **148**, 264, 273 (1925).

[2] H. **60**, 460 (1909); **118**, 267 (1922).

[3] H. **95**, 81 (1915); 111 (1917).

[4] R. Acc. Linc. **23**, 517, 612 (1914).

maldehyde. Another procedure, recently employed by Hunter and Dauphinee [1] as well as Edlbacher and Bonem for measuring enzyme action, is based upon the quantitative decomposition of the urea which has been set free by urease, and the colorimetric or else the titrimetric determination of the quantities of ammonia formed. These methods may possibly be preferable since they enable a comprehension as to the action of the arginase, together with the action of proteolytic enzymes, e.g., in enzyme preparates, which are subject to self-digestion. The dependence of arginase action upon the H-number has been tested by Hunter and Dauphinee and by Edlbacher and Bonem, and the optimum of the hydrolysis observed at pH = 9.5 to 9.9, corresponding to the alkalinity of a dilute solution of arginine carbonate. The decomposition of the arginine is almost complete under optimal conditions, its course corresponding to a considerable diminution of the mono-molecular reaction constant, according to Gross,[2] because of the strongly inhibiting action of ornithine. The unit proposed by Edlbacher and Röthler as a measure of the enzyme quantity is based upon the empirically obtained ratios between enzyme quantity and transformation. That enzyme quantity is designated as the "arginase-unit (A.E.)" which decomposes 10 ccm. of a 1 per cent solution of arginine carbonate in 60 minutes at 38° and pH = 9.5, with formation of a quantity of urea which gives 0.34 mg. of ammonia by the action of urease. The relations observed between the arginase amount and the transformation under the given conditions of determination, which correspond to a decrease in the relative reaction velocity with an increase in the enzyme amount, are in agreement for the enzymes of almost all animal organs. In one special case alone, that of the arginase of the kidney of fowls, did the investigation show a direct proportionality between the enzyme amount and the transformation; wherefore for this case the fixing of a special enzyme unit was regarded as necessary. This rather unusual phenomenon is ascribed to the lack of a specific inhibition body affecting the enzymic affinity the presence of which might also influence the reaction process. It recalls the observations regarding the influence of associated substances upon the affinity of yeast saccharase, as reported by Kuhn.[3]

[1] Prac. Roy. Soc. **97**, 209, 227 (1924).
[2] H. **112**, 236 (1920–21).
[3] H. **125**, 28 (1922–23).

The testing of the actions of arginase shows, according to Edlbacher and Bonem, that only the naturally occurring, dextrorotatory arginine is subject to scission and that other guanidine derivatives, as guanidine acetate or guanidine propionate, are not attacked. From the observation that neither the methyl ester of arginine nor the decarboxylated amino acid, agmatine, undergoes hydrolysis, and that in the dipeptide arginyl arginine only half the molecule does so, the conclusion is developed that only those arginine molecules are hydrolysed which carry a free carboxyl group.

A highly attractive problem in the field of enzymic research should be the proof by more exact methods of the distinction between arginase and histozyme, which is assumed but still not positively demonstrated. Or, on the other hand, the identity of both these enzymes might be demonstrated, which on the basis of the coincidence of their occurrence is not to be precluded without further evidence.

3. UREASE

Occurrence and Importance.—The observations regarding a bacterial decomposition of the urea in urine into ammonia and carbon dioxide, which go pretty far back, have played an important part in the discussion of the Liebig and the Pasteur views as to the essential nature of fermentation. However, the preparation of the active enzyme, urease, from bacterial material, to which alone its activity was formerly ascribed, was not accomplished until a comparatively recent date.[1] Furthermore, it was not until a considerably later date that attention was called to the general distribution of urease in the plant world, for whose protein metabolism the enzyme is of special significance. The enzyme is of particular significance since it acts in the decomposition of the intermediately formed urea, which is not eliminated, forming ammonia which is the necessary form of nitrogen for protein synthesis. Thus its occurrence has been found to be most pronounced in protein-containing seeds, especially those of the *Leguminosae*. Of these, the seeds of the soya bean as well as the jack bean have been found to be a particularly suitable source material for the production and investigation of this enzyme. It was first

[1] See M. Jakoby, Bio. Z. **84**, 354 (1917).

described in the soya bean by Takeuchi and Jonone [1] and is easily extracted from it by aqueous media. In the animal kingdom the enzyme is found only in isolated cases and in comparatively small quantities. The only known reaction which urease accelerates is the splitting of urea into ammonia and carbon dioxide. The most accurate methods for measurement of this reaction, among the great number of methods used, according to Lövgren,[2] are the determination of the unattacked substrate by xanthydrol, after the method of Fosse,[3] and the titration of the ammonia split off. van Slyke [4] recently described an accurate gasometric measurement of the CO_2 formed.

Hydrogen-Ion Dependence.—The optimal H-number for the action of urease was found by van Slyke and Zacharias [5] to correspond to $p\mathrm{H} = 7.0$. According to Lövgren,[6] it is dependent on the concentration of the urea, in that equal percentage variations in the urea concentration lead to equal percentage variations in the pH optimum. These important observations are illustrated in Fig. 12, which shows the pH optimum of the urease in dependence upon the urea concentration, expressed by the maximal possible quantities of NH_3.

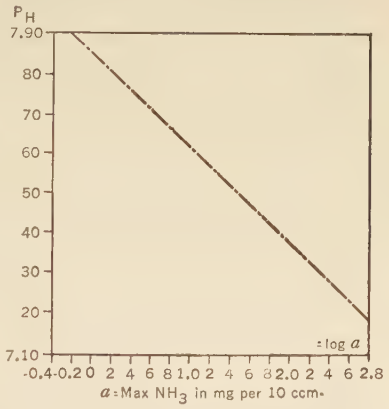


Fig. 12.—Urea Concentration and pH Dependence of Urease.

This finding is in contradistinction to the case of saccharase in which the pH is independent of the substrate concentration, and may be of special importance for a comprehension of the mechanism of urease action.

Supposed Activations.—The pronounced pH alteration of the reaction mixture occasioned by the gradually liberated ammonia

[1] J. College Agric. Imper. Univ. Tokyo **1**, 1 (1909).
[2] Bio. Z. **119**, 215 (1921).
[3] C. r. **158**, 1076, 1432 (1913); Soc. Biol. **77**, 129 (1914).
[4] J. Biol. Chem. **73**, 695 (1927).
[5] J. Biol. Chem. **19**, 181 (1914).
[6] Bio. Z. **137**, 206 (1923).

requires the use of higher buffer concentrations for the maintenance of a constant H-number. However, according to van Slyke and Zacharias these buffers considerably diminish the reaction velocity. According to Rona and György [1] the numerous statements in the literature regarding the specific activations of urease must be ascribed principally to an inadequate regard for the *p*H influence. These activations were first explained by Onodera [2] by the existence of a special co-enzyme, and were attributed by Jakoby [3] to the action of special "auxo-ureases," i.e., of amino acids and of the constituents of serum. It is readily seen that the presence of such amphoteric substances may decrease the *p*H alteration of the enzymic reaction mixture and therewith increase the transformation.

Kinetics.—Likewise the observations in the literature regarding the dependence of the transformation upon the substrate concentration appear in many cases to be distorted by an inadequate regard for the *p*H effects. Although from the investigations of Armstrong and Horton [4] as well as of Wester [5] the transformation appears to be independent of the quantities of urea employed, at least within certain limits, van Slyke and Cullen [6] have offered proof that with an increase of the initial concentration an absolute increase takes place in the substrate quantities transformed in equal times, up to a maximal value; the latter is true when the acidity is held constant and when dilute urea solutions are used, as is illustrated in Table XXXIV. From the numbers given, the dissociation constant of the urease-urea combination is calculated to be about 0.025.

No simple principle has yet been developed for the much-investigated reaction course of urea decomposition by urease. Van Slyke and Cullen, who divided the enzymic reaction into two partial processes, with different reaction velocities, i.e., into the formation and the breaking down of the urease-urea compound, have ascribed the reaction velocity to the sum of a linear and a logarithmic function of the transformation. But this theory,

[1] Bio. Z. **111**, 115 (1920).
[2] Biochem. J. **9**, 544 (1915).
[3] Bio. Z. **74**, 105 (1916); **114**, 152 (1921).
[4] Proc. Roy. Soc. **85**, 109 (1912).
[5] Ber. d. Deutsch. pharm. Ges. **30**, 163 (1920).
[6] J. Biol. Chem **19**, 141 (1914).

which had already been stated similarly by Henri [1] for the reaction course of enzymic cane-sugar hydrolysis, has not been confirmed by the later experimental researches of Barendrecht [2] and of Lövgren.[3] Thus Lövgren in particular has shown, in opposition to the earlier statements of Armstrong, Benjamin and Horton,[4] that the reaction course, at least for the initial region of the transformation, follows an equation of the first order, and that the product of the substrate concentration and the reaction velocity

TABLE XXXIV

TRANSFORMATION AND SUBSTRATE CONCENTRATION

According to Van Slyke and Cullen

Urea Concentration, Mol.	Milligrams NH_3, After 60 Min.	$K=\frac{10^4}{t}\log\frac{a}{a-x}$	$a\cdot k$
0.00625	0.99	45.0	1.4
0.0125	1.77	39.0	2.5
0.025	2.64	27.0	3.4
0.05	3.60	17.3	4.3
0.1	4.22	9.6	4.8
0.2	4.59	5.1	5.1
0.4	4.85	2.6	5.2
0.8	5.27	1.8	7.2
1.6	5.27	0.7	5.6

will be found constant within certain limits; whereas, for this magnitude within a single experiment a different course is observed according to the substrate concentration employed. This phenomenon, which is apparent from Table XXXV, is explained by Lövgren by the variation in the *p*H dependence of the enzyme upon decrease of the urea concentration, as results from the course of the experiment. According to this it will be of importance for the recognition of the mechanism of urease action to undertake the investigation of the enzymic reaction process, not with a constant H-number, but with one maintained at the optimum.

[1] C. r. **115**, 916 (1902).
[2] Rec. trav. chim. **39**, 1 (1920).
[3] Bio. Z. **119**, 215 (1921).
[4] Proc. Roy. Soc. B. **86**, 328 (1913).

TABLE XXXV

REACTION COURSE AND UREA CONCENTRATION

According to St. Lövgren

Substrate Concentration, Mol.	$a \cdot k$		Average Value (Corrected)
	Initial	Final	
0.00625	11	13	15
0.0125	16	17	21
0.025	21	29	31
0.05	31	41	37
0.1	34	44	43
0.2	34	34	35
0.2	39	39	39
1.6	38	27	36

Since this relation, which corresponds to the reaction equation $k = \frac{a}{t} ln \frac{a}{a - x}$, has been found to hold good only within certain narrow limits, Lövgren has attempted to establish a general time-equation for the urease action by purely empirical means. The applicability of this to the comparison of different enzyme preparations, however, appears to be doubtful.

Mechanism of Urease Action.—Various views have been advanced as to the mechanism of urease action; however, in no case do they appear to have sufficient experimental support. Thus, Armstrong and Horton [1] have assumed a direct decomposition of the urea in hydrated form, corresponding to the equation:

$$C\begin{matrix}(OH)_2 \\ (NH_2)_2\end{matrix} + 2H_2O \rightarrow C{\equiv}(OH)_4 + 2NH_3$$

whereas, Fearon [2] has postulated the formation of cyanic acid and ammonia as intermediate steps. While various investigators have stated that the hydrolysis of the urea proceeds to practical completion, and that no equilibrium is to be observed, Mack and Villars [3] as well as Kay [4] have called attention to small synthetic

[1] Proc. Roy. Soc. B. **85**, 109 (1912); **86**, 328 (1913).
[2] Biochem. Jl. **17**, 84 (1923).
[3] Am. Soc. **45**, 501 (1923).
[4] Biochem. Jl. **17**, 277 (1923).

activities of urease in concentrated solutions of ammonium carbonate, which should lead to the establishment of an equilibrium.

Preparative Purification.—With regard to the preparative methods for purification, it may be said that certain highly spectacular and interesting results have been recently communicated. Sumner [1] has reported, in fact, the production of a crystalline protein from jack-bean meal, which has given evidence of being identical with the enzyme itself. De-fatted jack-bean meal is extracted with a 31 per cent aqueous solution of acetone and the extract maintained at a low temperature. Colorless octahedra separate on standing, from which the mother liquor may be removed by centrifuging. The substance may be recrystallized without loss of activity and the recrystallized product has been reported to be 730 times more active than the most active jack-bean meal.

Euler and Brunius,[2] however, have been unable to repeat these results. Sumner attributes their failure to a low content of urease in the starting materials.

Euler and Brunius, as well as Sumner, have attempted the application of adsorption methods to the purification of this enzyme, but without very satisfactory results.

[1] J. B. Sumner, V. A. Graham, and C. V. Noback, Proc. Soc. Exp. Biol. and Med., **21**, 551 (1924); J. B. Sumner and V. A. Graham, ibid. **22**, 504 (1925); J. B. Sumner, Jl. Biol. Chem. **69**, 435 (1926); ibid. **70**, 97 (1926); J. B. Sumner and D. B. Hand, Jl. Biol. Chem. **76**, 149 (1928).

[2] Biochem. Zs. **183**, 1 (1927).

IV. CARBOHYDRASES

Among the carbohydrate-splitting enzymes, whose action relates to the scission of glucosidic linkages, we must discriminate between two large groups; the hexosidases, which catalyze the scission of the simple saccharides and glucosides; and the polyases, which effect the degradation of the higher carbohydrates, e.g., of starch or glycogen. A third group of enzymes, held to be specific, whose action consists in the rupture of glucosidic linkages in the nucleic acids and their degradation products, is as yet too little investigated to justify an authoritative decision as to their special existence.

1. HEXOSIDASES

a. Fructosidases: Saccharase

Special Characteristics.—The cane-sugar splitting invertin or saccharase, as found in yeast or in the intestine, may be regarded in general as the best investigated enzyme and as the enzyme of which the properties and mode of action are most intimately known. Contributing toward this special position of invertin are a number of factors which facilitate a description of its mode of action. For one thing, the analytical methods by which its action is measured are particularly simple, depending on the polarimetric determination of the change in rotation of cane-sugar solutions. Also its action is markedly independent of the influence of other materials. The relatively high stability of the enzyme is a salient advantage in its preparative treatment. In the general part of this book the most important points of information as to the kinetics and the reaction mechanism of enzyme action, as well as with respect to a series of influences which concern enzymic reactions, have already been mentioned and elucidated, saccharase being used as an example.

Measurement.—The following detailed descriptions will deal primarily with a characterization of the preparative behavior

of saccharase and a characterization of its special properties, as these have been derived from the researches carried on in recent years, particularly by the Willstätter and the Euler school. To this end it will suffice to introduce the preparative description of the enzyme with an explanation of the various scales employed for the quantity and degree of purity of the saccharase.

A more or less exact expression for the degree of purity of invertin was first established by O'Sullivan and Thompson.[1] These investigators characterized an enzyme preparation by the "time value," that is, the number of minutes required for 0.05 g. of a preparate to invert 4.0 g. of cane sugar to the point of zero rotation with respect to sodium light, 25 ccm. of a solution containing 1 per cent of NaH_2PO_4 at 15.5° being used. This point corresponds to a hydrolysis of 75.75 per cent. According to this method the time value decreases with increasing enzymic concentration.

From this definition of the enzymic purity-degree, Willstätter and Racke[2] first derived the "quantity-time-quotient (M.Z.Q.)" as a measure for the magnitude of enzyme quantity, i.e., the quotient of the enzyme material obtained in any form and its action-time as measured by the time-value. This value, which is directly proportional to the enzyme quantity, represents an additive magnitude.

Willstätter and Kuhn[3] have replaced the expression M.Z.Q. by the more readily apparent measure, the "saccharase unit (S.E.)," namely, the amount of enzyme of the time-value 1 in 50 mg. of invertin-containing substance acting under the conditions of the definition of O'Sullivan and Thompson. The purity degree of the enzyme is expressed by "saccharase-value (S.W.)," the number of saccharase units in 50 mg. of preparate, it designates, accordingly, the reciprocal of the time-value and rises with increasing enzymic concentration. The saccharase-value bears a simple, calculable relation to a rational measure, the "inversion capacity," $I_f = \frac{k \cdot g\ \text{Sugar}}{g\ \text{Preparate}}$, introduced by Euler and Svanberg[4] for designating the degree of purity. According to Willstätter and Kuhn the scales for the saccharase-unit and for the saccharase-value are to be calculated to a saccharase of average affinity, when

[1] Soc. **57**, 834 (1890).
[2] A. **425**, 1 (1921).
[3] B. **56**, 509 (1923).
[4] H. **107**, 269 (1919).

comparing enzyme preparates with different affinities; this is because of the varying affinities of saccharases to cane sugar. The average affinity constant for a saccharase is equal to 50 (dissociation constant of the saccharase-cane-sugar compound = 0.020). The reduced saccharase units are expressed by the relation $S.E._{red} = S.E.\frac{n+k}{n+0.02'}$, in which n represents the normality of the cane-sugar solution in which the reaction velocity is determined. This calculation can be dispensed with, however, when following the enzyme quantities in the course of the preparative work from a given source material, wherein the affinity remains the same.

Occurrence.—The cane-sugar splitting enzyme is found in both the animal and vegetable worlds; it is particularly widely distributed in the latter. Aside from its most often studied presence in the cryptograms, e.g., in yeast, it is to be remarked here that the enzyme, according to the statements of Kastle and Clark,[1] is found not only in the cane-sugar storing parts of the plants, e.g., in the sugar beet, but in general wherever the higher carbohydrates, such as inulin, serve as reserve substances. The distribution of the enzyme in the animal world is comparatively limited. Beside the honey vesicle and the saliva of sugar-inverting insects, saccharase is found chiefly in the intestine, in which it was discovered by Bernard.[2] According to the researches of Bierry [3] and of Euler and Svanberg [4] it is to be assumed that the enzyme is not given off from the cells of the intestinal mucous membrane into the secretion, but that the transformation of the cane sugar takes place in the gland cells themselves; in the pure fistula juice of the intestinal glands no inverting action is noted. According to Euler and Svanberg, the animal enzyme differs from the saccharase of yeast by virtue of its lower stability and by the dependence of its action upon the H-number, whose optimum for intestinal invertin is found at pH = 5 to 7, whereas, that for the enzyme of yeast occurs at pH = 4.5; the activity of the plant enzyme at pH = 7 amounts to only about one-third of the optimal.

Specificity.—According to Kuhn [5] the animal saccharase and likewise that of *aspergillus oryzae* also differs from the saccharase of yeast with respect to its specificity. Whereas, the enzyme of

[1] Am. **30**, 422 (1903). [2] Lec. sur le Diabete, Paris, 1887, p. 259.
[3] Bio. Z. **44**, 415 (1912). [4] H. **115**, 43 (1921). [5] H. **129**, 57 (1923).

yeast, according to Willstätter and Kuhn [1] hydrolyzes the trisaccharide raffinose also as well as cane sugar, no action is observed with animal or taka-saccharase upon raffinose or other fructosides. Recent observations by Nelson and Papadakis [2] would indicate, in fact, that there is a distinction between the yeast-enzyme hydrolysing raffinose and that hydrolysing saccharose. Their experiments have developed conditions of heat inactivation which affect the two hydrolysing actions in a different manner. Kuhn has reported that the action of the yeast enzyme is inhibited only by fructose, and that of the enzyme of *aspergillus oryzae* on the other hand only by glucose. This phenomenon, according to Kuhn, is connected with the conclusion that the hydrolysis of cane sugar by the two enzymes proceeds through different kinds of intermediate reaction products, namely, by an attachment of the enzyme in one case upon the fructose-residue and in the other case upon the glucose residue of the disaccharide. On this basis, a differentiation between fructo- and gluco-saccharases appears to be feasible, according to the researches of Kuhn and Münch [3] as well as those of Kuhn and Grundherr.[4] This is in opposition to the views held by Euler and Josephson [5] according to which the action of the enzyme takes place in all cases by a simultaneous attachment on both sugar residues. Also the observations of Nelson and Anderson [6] do not confirm the reported differences in inhibition by glucose and fructose.

Liberation of Yeast Saccharase.—The preparative separation of saccharase, to which Willstätter and his collaborators, as well as Euler, have devoted a great number of investigations, has hitherto been carried out only with the enzyme of yeast. While Fischer [7] has advanced the view that the cane-sugar splitting enzyme passes into solution upon extraction of the fresh yeast with water, Willstätter and Racke [8] have shown by quantitative controls that the isolation of the enzyme from yeast does not consist in

[1] H. **125**, 28 (1922–23).

[2] Am. Soc. in press.

[3] H. **150**, 220 (1925); H. in press.

[4] B. in press.

[5] H. **132**, 301; **134**, 50; **136**, 62 (1924); **143**, 79; **149**, 71 (1925); **152**, 31, (1925-26).

[6] J. Biol. Chem. **69**, 443 (1926).

[7] B. **27**, 3479 (1894).

[8] A. **425**, 1; **427**, 111 (1921).

a simple solution process and that in the usual process of grinding and pressing or of lixiviation with water only a fraction of the saccharase goes into solution. It has further been shown that in the various processes of rapid or gradual autolysis of the fungus, which lead to preparative yields of enzymic substance, the solution of the saccharase is to be ascribed to an enzymic process—to a liberation of the enzyme, which constitutes a precisely definite, partial process in the general enzymic decomposition of the yeast substance. The observation that with the emptying of the yeast cells by means of proteolytic enzymes no saccharase goes into solution, and that on the contrary its decomposition by the action of carbohydrate-splitting enzymes effects the liberation of the invertin, appears to permit the conclusion that the enzyme in the yeast cell is protected by an attachment in the cell membrane composed of polyoses. Its liberation from the membranes, according to this, is accomplished either by enzymic dissolution, which consists in the decomposition of the polyoses, or by mechanical action, i.e., by complete destruction of the cell-structure.

Autolysis Procedures.—The conditions of yeast autolysis are of great significance for the quantity and for the nature of the concomitant substances which go over with the enzyme into the autolysate. The researches of Willstätter [1] have led to the development of four different autolytic processes, whose special fitness for the preparative purification of invertin is evident from the accompanying summary. This summary shows together with the method the improvement attained in the enzymic concentration of the autolysates:

1. The process of a 4 to 7-day autolysis at room temperature by action of toluol on the yeast diluted with water, according to Hudson.[2] By this means an enzyme accumulation double that of the concentration in the dry yeast is attained.

2. The similar process of neutral autolysis of diluted yeast,[3] namely, with neutralization of the acids formed. This yields enzyme solutions of an approximately 3-fold concentration.

3. The process of rapid autolysis at neutral reaction,[4] consisting in a

[1] Compare R. Willstätter, K. Schneider and E. Bamann, H. **147**, 248 and particularly p. 264 (1925).

[2] Am. Soc. **30**, 1564 (1908); **36**, 1566 (1914).

[3] R. Willstätter and F. Racke, A. **425**, 1 and particularly p. 54 (1921).

[4] R. Willstätter and K. Schneider, H. **142**, 257 and particularly p. 266 (1924–25).

1 to 2-day action of chloroform upon undiluted yeast and subsequent dilution and neutralization. The purity degree of the autolysate is similar to that observed in the second process.

4. The process of fractional autolysis of undiluted yeast [1] by killing with toluol with neutralization, rapid separation of the invertin-poor liquefaction juice formed after about an hour and continuation of the autolysis up to 1 day. This process, which has been found of particular value in the elaboration of enzyme-rich yeasts, leads to an increase of enzymic concentration of about 4 to 5 times that of the source material.

According to Willstätter, Schneider and Wenzel [2] marked differences in the qualitative composition of the autolysates are noted only between the rapidly autolysed, undiluted yeast and the slower autolysed, diluted material, the yeast being far more thoroughly exhausted in the latter. The degradation enzymes, which, because of the more energetic killing of the undiluted yeast, have a weaker action in the gentler killing with the diluted yeast, effect a more selective liberation of the invertin and a decreased decomposition of the enzyme complex. The quantity relation of the saccharase to other enzymes and to their decomposition products is more favorable in the case of the undiluted autolysis. The concentration of the enzyme in the autolysates is further considerably augmented by the process described by Willstätter, Lowry, Jr., and Schneider [3] for the specific enrichment of invertin in the yeast. This is based upon a fermentation procedure with minimal cane-sugar concentration, the experimental principles of which have already been fully explained in the general part of this book. By the combined application of these methods and by the perfecting of the liberation process the richest enzyme extracts are obtained, the purity-degree of which corresponds to saccharase values of 0.5 to 1.0, compared to an enzymic concentration of S.W. of about 0.03 to 0.05 in the original fungus material.

Preparative Purifications.—The preparative purification of invertin from the yeast autolysates has been carried out in a number of cases by Willstätter. Meanwhile the knowledge of invertin, gained in the first works on the subject, permits no generally valid prescriptions and directions, but merely furnish a number of examples. The behavior of saccharase upon purification, with respect to precipitation as well as to adsorption

[1] R. Willstätter, K. Schneider and E. Bamann, as before.
[2] H. **151**, 1 (1925–26).
[3] H. **146**, 158 (1925).

methods, is in large measure dependent on the nature and the age of the yeast extracts employed, that is, upon the quantity and nature of the associated materials. It may be added that the choice of the processes selected for enrichment of the enzyme content is influenced by the purpose of the purification, i.e., the object may be exclusively an increase in purity-degree or merely the elimination of certain accessory substances. The older examples of the purification of the enzyme are far surpassed by the newer investigations, whose advanced improvements are based on the use of fractional autolysis of yeast particularly rich in invertin, these improvements relating to the number and duration of the operations required for purification and to the reproducibility of the process. Nevertheless, it might be of interest here to describe briefly the most important results of the older methods, especially since they have contributed to the development of the preparative method.

Willstätter and Racke,[1] whose starting point was yeast autolysates according to Hudson, purified the saccharase by separating out impurities with kaolin and by fractional adsorption from the mother liquor with alumina in an acid reaction with the addition of acetone. It developed that, after acidification of the enzyme in the ammoniacal elutions of the alumina adsorbate, it could be taken up by kaolin. Consequently the adsorption behavior in crude solutions, which was explained by Michaelis [2] in the sense of a predominantly acid nature of the enzyme, was in reality disguised by concomitant substances. The significance of the adsorption of the enzyme on kaolin was manifested by the observation that in the elutions of the adsorbates, whose best example is characterized by the saccharase-value 1.0, the enzyme can be obtained free from yeast gum, a carbohydrate which has frequently been identified with it. The investigation conducted by Willstätter, Graser, and Kuhn [3] advanced preparative and analytical knowledge through the discovery that the invertin from the aged yeast autolysates is precipitated by lead salt, whereas in the fresh extracts no precipitation of the enzyme is observed. Accordingly, by the use of fractional precipitation with lead in the case of adsorptively pre-purified enzyme solutions, it was found that a method was provided for the separation of phosphorus-containing impurities. The tenacious association of these

[1] A. **425**, 1 (1921). [2] Bio. Z. **7**, 488 (1907–08). [3] H. **123**, 1 (1922).

impurities with the enzyme had led Euler and Svanberg [1] to consider that they were involved in the composition of the enzyme. The purest preparate obtained, which was prepared by a combination of alumina adsorption and fractional lead precipitation, corresponded to the saccharase value 5.0.

Important progress in methods was made by Willstätter and Wassermann [2] with respect to the use of kaolin for the adsorption of saccharase. They found that the disturbing influences of the concomitant substances with which the enzyme is associated in the crude yeast extracts and which affects its adsorption by kaolin, are eliminated by very great dilution and by suitable acidity, so that the saccharase can be adsorbed by kaolin directly from the yeast autolysates. Willstätter and Schneider [3] were further able to show that this operation is also successful without dilution and with any desired autolysates, provided that care is taken to employ a strongly acid reaction. By the systematic use of fractional adsorption with alumina and by assiduous searching for the conditions most favorable at the given time for the selective adsorption of the enzyme, Willstätter and Schneider [4] have obtained a further increase of the enzymic concentration with the saccharase-value 6.66. The method of adsorption was so conducted that the behavior of the enzyme approached more and more nearly the adsorption behavior of a pure substance, which was observed at the attainment of a saccharase value of about 5. But analysis of the preparate in this condition led to the discovery that the enzyme, though it behaved in the adsorption like an integral substance, was associated with an apparently large tryptophane-containing, peptide complex, which appears to shield the enzyme from inactivation and for whose separation the given preparative directions appear to be inadequate.

The degree of purity attainable by the older processes involving fractional adsorption and precipitation of the invertin from the extracts of dilute autolysis of ordinary yeast has not been markedly increased by the newer investigations, even with considerably improved source material. The expectation that after a notable increase of the invertin content in the fungus material and after the perfecting of the liberation process, the application of the adsorption method would lead to invertin preparates of

[1] H. **112**, 282 (1921).
[2] H. **123**, 181 (1922).
[3] H. **133**, 193 (1923–24).
[4] H. **142**, 257 (1924–25).

much higher enzymic concentration, has not been realized. It is true that Willstätter, Schneider and Wenzel [1] by fractional autolysis of yeast, have succeeded in obtaining extracts of which the purity-degree corresponds to saccharase values of 0.5 to 1.0, and which may be brought by simple dialysis to an enzymic concentration of a saccharase value of 2.5; the enzyme content of their original yeast being very high, e.g., increased to about 15 times the ordinary amount. However, the adsorption of these yeast extracts with alumina and with kaolin has again led to an augmentation in the degree of purity, which considerably transcends anything possible heretofore. "The enzyme with a saccharase value of about 5 is united with its coadsorbent in such a tenacious aggregate that, in its adsorptive behavior toward alumina, it behaves as a homogeneous substance." The improvement of the new process with respect to the older ones consists in the possibility of obtaining enzyme preparates of a high purity-degree from the yeast in a shorter working time. While the new yeast extracts of a high enzymic concentration are not fitted for the use of kaolin adsorption, it has been possible to obtain, e.g., by a single adsorption of the enzyme on alumina and subsequent dialysis and electro-dialysis, preparates of a saccharase value of about 7.3. Also, according to Willstätter and Bamann,[2] the quantity relation of the saccharase to the other enzymes of the autolysates may be more favorably developed by separating out the malt-sugar splitting enzyme through selective adsorption with alumina of definite composition, or else by the selective elution of alumina adsorbates of both enzymes; that is, by the action of acid phosphate solutions, only the saccharase is dissolved out of the mutual adsorbate.

Further improvement in technique has been realized by fractional precipitation of the invertin solutions (pre-purified with alumina) by means of tannin at a low temperature. This has led to preparates having a saccharase value of 9.4. These methods are exceeded in both preparative and general importance by the use of a process of fractional adsorption on an adsorbent which is first produced within the enzyme solution itself. Thus it has been possible by means of the partial formation of an adsorptive precipitate of lead phosphate to raise the saccharase value in the elution of the last fraction to about 10. The important observation has

[1] H. **151**, 1 (1925–26). [2] H. **151**, 273 (1925–26).

further been made that this method permits a positive separation of the enzyme from its inactivation products; partially inactivated enzyme solutions can be repurified to the same degree of purity as their original value.

Composition of Saccharase Preparates.—Analysis of the purified enzyme preparations obtained (having different degrees of purity) has indicated that saccharase, like other enzymes, belongs neither to the carbohydrate nor the proteolytic class of substances. Still it has not yet been possible to trace the nature of the enzyme to definite, chemically defined, structural units. The older views of Fodor [1] or of Euler and Svanberg [2] according to which the invertin was to be identified with yeast gum or with a phosphorus-containing constituent of the yeast, have proved to be untenable, following the complete separation of these materials from the enzyme. Euler and Josephson [3] meanwhile in later researches have advanced the opinion that saccharase is to be considered as a protein-like substance, and that its activity is connected in the closest manner with that component of the enzyme which is designated as the "protein part"; according to the theory, it is this portion which effects the degradation of the substrate. These conclusions are derived from the behavior of saccharase with respect to trypsin, by which it is attacked with difficulty, with an invariable loss of activity. They are also derived from a proportionality observed between the enzymic concentration and the nitrogen and sulfur content of the preparates. They are extended by later data as to a wholly specific manner of construction of the enzyme proteins, characterized by a high content of tryptophane, which seems to increase with the enzymic concentration.

These special views are not confirmed by the researches of Willstätter. For one thing it has been found [4] that a proportionality between the nitrogen content and the enzymic degree of purity is not invariably observed. Also, the sensitive protein reactions which appear more or less clearly in some of the purer enzyme preparates are lacking in others. Furthermore, it has been found

[1] Das Fermentproblem. Dresden and Leipzig, 1922.

[2] H. **112**, 282 (1921).

[3] B. **56**, 446, 1097 (1923); **57**, 299, 859 (1924); H. **138**, 11, 38 (1924); **145**, 130 (1925).

[4] R. Willstätter and K. Schneider, H. **133**, 193 (1923–24).

possible [1] to make the saccharase (pre-purified under certain given conditions) completely free from its original content of tryptophane, by fractional adsorption with kaolin, though this entails, it is true, a certain loss of activity; the enzyme appears to be stabilized by intermixed tryptophane-containing peptides. The proof that the content of protein cleavage products is not to be regarded as significant for the composition of saccharase has been convincingly demonstrated by Willstätter, Schneider and Wenzel.[2] They have established the fact that it is also possible to obtain the enzyme entirely free from tryptophane peptide as well as from tyrosine peptide by fractional adsorption with lead phosphate. Moreover, the content of tryptophane alone does not appear to be of necessity for the stability of saccharase.

Whereas, Euler and Josephson [3] proceed from the assumption that the colloidal bearer of saccharase is to be definitely distinguished from other concomitant substances contained in the source material, according to Willstätter, Schneider and Wenzel, it is impossible to distinguish the colloidal concomitants of the enzyme, such as yeast-gum, nucleic acids, or peptides, from other associated materials in their action on the enzyme. Since the enzyme can be completely freed from any of these materials by various purification procedures, it would follow that they are without significance to the composition of the enzyme itself, although in each particular case they may be of influence in maintaining enzymic activity. Indeed the actual separation of the individual saccharase molecule, the specifically active group itself, from all its protective colloids, has never been accomplished while the enzyme yet retained its activity. However, the experimental observations which have been made, with respect to the behavior of the enzyme during the separation of the various individual protective substances, lead to the conclusion that the nature of the colloidal carriers is variable and that the aggregates of the enzymes are capable of variation.

b. α-Glucosidases: Maltase

Occurrence.—The most important enzyme rupturing α-glucosidic linkages is maltase, which was first described in yeast by

[1] The same, H. **142**, 257 (1924–25).
[2] H. **151**, 1 (1925-26).
[3] H. **145**, 130 (1925).

Bourquelot [1] and which is widely distributed in animal and in plant organisms. It is usually found in conjunction with the starch-splitting enzyme, amylase, whose action results in the formation of maltose; the combination of these two enzymes acting together accomplishes the degradation of carbohydrates to grape-sugar. The enzyme of animal organs and secretions has not been studied a great deal and its description also in the seeds of the higher plants has been restricted because of a protective stratification which hinders the dissolution of the enzyme and because of its low degree of stability. On the other hand, numerous works have been devoted to the investigation of maltase in yeast, where it occurs abundantly.

Hydrogen-Ion Dependence.—The *p*H optimum of the maltase action of yeast, which, according to Rona and Michaelis [2] as well as according to Willstätter and Steibelt,[3] lies between *p*H = 6.0 and 6.8, differing somewhat with the substrate employed, has been determined by Willstätter and Bamann,[4] using more suitable buffers, as ranging from 6.75 to 7.25, therefore from a very weak acid to a weak alkaline reaction.

Kinetics.—The kinetics of maltose cleavage, which according to the researches of Willstätter, Oppenheimer, and Steibelt,[5] has been found to vary from yeast to yeast and from substrate to substrate, follows but rarely the reaction equation of the first order. In agreement with the statements of the older literature, the reaction ordinarily proceeds more slowly than the mono-molecular. This phenomenon has been attributed by Willstätter, Kuhn and Sobotka [6] to varying affinities of the enzyme, the cause of which is not known. Important contributions have been made in this direction by the research of Willstätter and Bamann. It has been shown that the reaction process of malt sugar cleavage is in high degree dependent on the extent of purity of the enzyme.

The kinetics observed with crude yeast and its crude extracts is no longer valid for the adsorbates of the enzyme on alumina or for the elutions obtained therefrom. Even when a part of the

[1] J. de l'Anat. et Physiol. **22**, 162 (1886).
[2] Bio. Z. **57**, 70; **58**, 148 (1913).
[3] H. **115**, 199 (1921).
[4] H. **151**, 242 (1925–26).
[5] H. **110**, 232 (1920); **115**, 199 (1921).
[6] H. **134**, 224 (1923–24).

enzyme is adsorbed by the introduction of alumina into its solution, the total amount of maltase in the solution, as well as in the elutions prepared from the adsorbate, follows another time-law, i.e., its action becomes slower. This phenomenon is based, according to Willstätter and Bamann, not upon a destruction of the enzyme (which has become purer) in the course of its reaction, since even in the purer forms the product of the enzyme quantity and the reaction duration are constant for the same degree of cleavage; it is attributed rather to the separating out of a substance whose presence may counteract the inhibition by the scission product, glucose. These alterations of the reaction kinetics in one direction or another may be observed also in the filtration of aged autolysates or upon the standing of the filtered solutions, in which the reaction-promoting substance appears to be re-formed. The observed alterations of the enzymic activity present greater difficulties to the exact comparison of the maltase quantities in different degrees of purity.

Scale for Measurement.—Thus the "apparent maltase unit (M.E.)" has been established as a measure of the enzyme quantity. It represents that quantity of enzyme which splits half of 2.5 g. maltose hydrate in 50 ccm. of solution at pH = 6.8 and 30° in 1 minute. This expression, however, needs to be supplemented by the data obtained for the kinetics observed at the given time.

The maltase value (M.W.), i.e., the number of M.E. in 1 kg. of the preparate, serves as a measure of the degree of purity.

Specificity.—According to Willstätter, Kuhn and Sobotka there are no grounds for the hypothesis of different absolute specific enzymes for the hydrolysis of maltose and for the other α-glucosides,—they seem accordingly to be split by the same enzyme. The enzyme-value-quotients obtained with due regard for the existing dissociation constants of the maltase-glucoside combinations, i.e., with equal concentrations of the reaction intermediate products, are in fairly close agreement for the comparison of the hydrolysis of maltose, α-methyl and α-phenyl glucoside by different yeast races. This is evident from Table XXXVI.

Maltase Solutions from Yeast.—The determination of the malt sugar splitting enzyme in the living yeast itself, which presented great difficulties to the earlier researches,[1] has been perfected and

[1] See G. H. Morris, Proc. Chem. Soc. 1895, p. 46; E. Fischer, B. **27**, 2985, 3479 (1894); **28**, 1429 (1895).

established quantitatively by Willstätter and Steibelt[1] and by Willstätter and Bamann.[2] According to this it appears that the maltase, in contrast to the cane-sugar splitting enzyme, is sufficiently accessible to the sugar entering the cells only after the marked alterations which take place upon killing. For the exact analysis of the enzyme in the yeast it is necessary merely to restrict the killing of the fungus to a short period of time and to provide, by suitable buffering, for a neutralization and elimination of the acids which appear during the autolysis. The agreement of the enzyme quantities which are obtained according to several methods of determination, either by rapid killing of the fungus, with cell-poisons such as acetic ester, toluol, chloroform, or by osmotic destruction, i.e., by the action of diammonium phosphate or sodium chloride, is further extended by the observation that it is possible to transfer exactly as much maltase into the solution by autolysis as is found in the analysis of the yeast itself.

TABLE XXXVI

Enzyme-Value-Quotients for Various Yeast Races

Yeast Race	Quotient	
	α-Methyl Glucoside-Maltose	α-Phenyl Glucoside-Maltose
Lion Brewery yeast	0.21	5.1
Hofbräu yeast	0.18	6.1
Copenhagen yeast	0.23	

The isolation of maltase from yeast by leaching out with water was possible, according to the earlier researches by Lintner,[3] and Hill[4] only after a preliminary drying of the yeast, and not by the ordinary process of autolysing the fresh yeast materials. The observed difference between the behavior of saccharase and maltase was ascribed to a difficult solubility of the maltase, or to a

[1] H. **111**, 157 (1920).

[2] H. **151**, 242 and particularly p. 253 (1925–26).

[3] Z. f. ges. Brauw. 1892, p. 106; C. J. Lintner and E. Kröber, B. **28**, 1050 (1895). E. Fischer, B. **27**, 3479 (1894); **28**, 1429 (1895); H. **26**, 60 (1898).

[4] Soc. **73**, 634 (1898); **83**, 578 (1903).

prevention of its diffusion through the cell membrane. Willstätter, Oppenheimer and Steibelt [1] have subsequently shown that the acid, which appeared during the killing of the living fungus with cell-poisons, destroyed the more sensitive maltase, and, further, that it was possible to obtain autolysates of high maltase content by simply neutralizing the acids formed. The liberation of the enzyme from the yeast cell was then improved and conducted more specifically by Willstätter and Bamann, by the process of fractional autolysis of the yeast. This differentiates between the liquefaction of the cells and the enzymic process of liberation which set in after the killing.

It has been shown that rapid killing of the yeast in undiluted condition is more advantageous in the production of maltase as also in the production of invertin. This is conveniently carried out with ethyl acetate or with pulverized diammonium phosphate. Not until then does the process of dissolution of the enzyme from the cell-content begin, this being carried out with dilution, by the use of acetic ester, and with neutralization. After the lapse of $\frac{1}{2}$ to 1 day the total maltase of the yeast together with only a fraction of the original yeast mass is found in the autolysates. Still purer maltase solutions are obtained after separating the liquefaction juices which first appear. Comparison of the dissolution velocity of maltase and of saccharase led to the surprising discovery that the maltase was transferred, not with more difficulty, but more rapidly from the cell into the solution.

Separation from Saccharase.—In contrast to saccharase, maltase is much more unstable, even in the crude yeast extracts. Willstätter and Bamann advise therefore that the enzyme solutions be maintained and worked at a constant temperature of 0°. It is because of this instability of the enzyme that systematic efforts to purify it have not yet been made. It need only be remarked here that, according to Willstätter and Bamann,[2] maltase and saccharase exhibit such appreciable differences in their adsorption behavior towards alumina that it is possible to separate them by this means. The separation of the two enzymes by adsorption, for which the use of an alumina gel of the formula AlO.OH has been found most suitable, does not appear to be

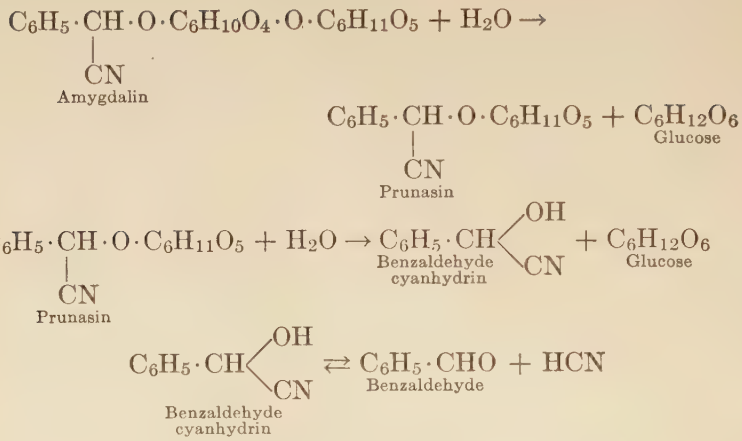
[1] H. **110**, 232 (1920). [2] H. **151**, 213 (1926).

based on a difference in the acid or basic properties of the enzymic individuals. The adsorbent used, whose selective action is greatest, possesses the least pronounced acid or basic character among the types of alumina tested. And yet adsorption processes take up the total amount of maltase, which, in fact, is found in an enzymically integral condition in the alkaline solutions of the adsorbate. On the other hand, the observation of Willstätter and Bamann concerning the selective elution of saccharase from mutual alumina adsorbates of both enzymes by the action of primary phosphate is to be considered in an electro-chemical sense. (In this elution process the maltase alone is left behind.)

c. β-Glucosidases: Emulsin

Reaction Course of Amygdalin Cleavage.—The classic reaction of the splitting of the natural glucoside amygdalin found in bitter almonds into two molecules of β-glucose, benzaldehyde and hydrocyanic acid is to be attributed to the combined action of three enzymes. This has hitherto been collectively considered under the term, emulsin action. The first step in the reaction consists in the splitting off of one molecule of glucose from the amygdalin disaccharide by the so-called amygdalase, which has frequently been identified with gentiobiase. This is a glucosidase found in yeast and effects, for example, the scisson of gentiobiase, 1.6 β-glucosido—β-glucose. In the second step of the reaction, the rupture of the glucosidic linkage between the benzaldehyde-cyanhydrin and the second molecule of β-glucose takes place; it is to be attributed to a special β-glucosidic enzyme, prunase [1] or β-phenyl-glucosidase. For the third reaction stage, finally, the splitting of the benzaldehyde-cyanhydrin into its components, the existence of an oxynitrilase is assumed. Its properties appear to be but imperfectly established as yet and its special synthetic actions have already been treated in the general portion of this book. The equations involved in these reactions may be represented as follows:

[1] H. E. Armstrong, E. F. Armstrong and E. Horton, Proc. Roy. Soc. B. **85**, 359 (1912).



According to the important observations of Fischer [1] the first phase of the reaction is the cleavage into prunasin and β-glucose; and this only is also effected by a β-glucosidase contained in the yeast, whereas, the prunasin-decomposing β-glucosidase appears to be lacking in the fungus. This finding, together with the earlier theory that yeast produces only α-glucosidase, was taken for a long time as supporting the hypothesis that the second glucose molecule in amygdalin corresponded to an α-glucosidic linkage. The complexion of this situation, however, has been changed somewhat by the synthesis of amygdalin from gentiobiose, β-glucosido—β-glucose, and with the recognition of its β-glucosidic structure. However, it cannot as yet be positively adjudged whether two specific β-glucosidic enzymes take part in the decomposition of the amygdalin of bitter almonds or whether it must be assumed that the splitting off of both molecules is accomplished by one and the same enzyme, the specificity of which is extended beyond that of the yeast enzyme. Also it is not yet known whether the enzyme of yeast, which has been termed amygdalase, is to be identified with a gentiobiose splitting enzyme likewise found in yeast.

With respect to the β-glucosidase of emulsin, Willstätter and Oppenheimer [2] by quantitative measurements, first made the discovery that it is capable of splitting not only amygdalin, but also other β-glucosides, such as helicin, salicin, and β-phenyl glucoside. Aliphatic β-glucosides such as β-methyl glucoside also

[1] B. **28**, 1508 (1895). [2] H. **121**, 183 (1922).

belong among its specific substrates, according to the investigations of Willstätter, Kuhn, and Sobotka.[1] This fact was demonstrated by the agreement between the glucosidase-value-quotients, obtained by taking into consideration the varying affinities, i.e., obtained with equal concentrations of the enzyme substrate compound.

Kinetics.—The time course of the hydrolysis of simple β-glucosides, such as arbutin, β-methyl glucoside, and β-phenyl glucoside —but not, on the contrary, of helicin—follows the equation of the first order, according to the reports of Hudson and Paine [2] and of Willstätter and Oppenheimer.[3] On the other hand, according to the observations of Willstätter and Csanyi [4] the mono-molecular reaction equation no longer holds good for the more involved process of amygdalin cleavage; a steady diminution of the reaction constants is observed. In this process, according to Auld [5] as well as according to Armstrong and Horton, glucose formation exceeds the liberation of benzaldehyde and hydrocyanic acid, whereas, the partial process of the decomposition into glucose and prunasin, which is catalyzed by yeast enzyme, follows a mono-molecular course, according to Willstätter and Steibelt.[6] It may be added that Willstätter and Csanyi have also found that the *p*H optimum of amygdalin cleavage and of prunasin cleavage are appreciably different, corresponding to a hydrogen number of 6.0 or 4.4. With a longer duration of the experiment, the optimum for the total cleavage process is displaced in the direction of a more acid reaction.

Measurement.—As a measure of the concentration of the enzyme preparate in controlling the preparative purification of emulsin, Willstätter and Csanyi used the time value of the 50 per cent cleavage of the glucoside. The time value, for example, is the number of minutes required by 1 mg. of the preparate to liberate about 50 per cent of the theoretical monose quantity from 0.1 g. of amygdalin, under the conditions of the experiment. The liberated sugar is determined by reduction after separation of the

[1] H. **123**, 33 (1923).

[2] Am. Soc. **31**, 1242 (1909).

[3] See the somewhat divergent reports by K. Josephson, H. **147**, 1 and particularly p. 33 (1925).

[4] H. **117**, 172 (1921).

[5] Soc. **93**, 1251, 1276 (1908).

[6] H. **134**, 224 (1923–24).

hydrocyanic acid. A "quantity-time-quotient" served as a measure of the enzyme quantity, as in the case of saccharase. These expressions have been replaced by Willstätter, Kuhn and Sobotka, by the more obvious measures of the β-glucosidase value and the β-glucosidase unit.

Preparative Separation.—The experiments described in the older literature with respect to the preparative isolation of the enzyme from bitter almonds made no attempt to separate the concomitant enzymes.[1] Ohta [2] as well as Helferich,[3] by digestion with trypsin, obtained protein-free preparates from the almonds or from their extracts, pre-purified by means of alcohol and dialysis. Willstätter and Csanyi [4] improved the yields in the aqueous extracts of the oil-free almond powder by the action of dilute alkali, which broke down the adsorptive binding of the enzyme to the insoluble substance; purification of the extracts by acetic acid and the repeated precipitation of the enzyme by alcohol led to an increase of about 40-fold in the enzymic concentration, corresponding to a time-value of 10 in the purest preparates; these still contained small amounts of protein substance, however.

Still purer preparates (β-Gl.W. = 81) have been obtained recently by Josephson [5] by adsorption on kaolin and dialysis of the ammoniacal elutions.

We must here forego a discussion of other β-glucosidases, such as cellobiase, which has been observed in many molds and seeds, or of gentiobiase, which has also been observed in yeast, particularly since the special existence and the specificity of these enzymes appears to be as yet inadequately explained.

d. Galactosidases: Lactase, Melibiase

The specificity of the galactoside-splitting enzymes has not as yet been sufficiently studied. According to our present knowledge we must differentiate between at least two enzyme groups, for one of which, lactase, the milk-sugar splitting enzyme may be regarded as a characteristic example, while melibiase will serve

[1] See on the contrary K. Josephson, B. **58**, 2726 (1925).
[2] Bio. Z. **58**, 329 (1914).
[3] H. **117**, 159 (1921); **128**, 99 (1923).
[4] Same as before.
[5] B. **59**, 821 (1926).

to represent the other, since their substrates differ in structure, lactose being a 5-galactosido-glucose and melibiase a 6-galactosido-glucose.

A lactose-splitting enzyme is found particularly in the milk-sugar yeasts, and in Kaffir grains, and also in mold fungi such as *aspergillus oryzae*; its action, further, is to be noted in bitter almonds. The enzyme of the milk-sugar yeasts appears to be a β-galactosidase, since according to the statements of Fischer and Armstrong [1] it also hydrolyses simple β-methyl galactoside. The view of H. E. Armstrong, E. F. Armstrong and Horton [2] according to which the enzyme of bitter almonds must be differentiated from this, since the action of the latter is inhibited only by galactose and of the former only by glucose, still requires more exact confirmation, even though Willstätter and Csanyi [3] also found appreciable differences in the *p*H-optima of the two enzymes.

Melibiase differs chiefly from lactase in its occurrence; it is found, in contrast to the other, not only in almonds but also in bottom-fermentation yeast, whereas both enzymes are lacking in top-fermentation yeast. It is not yet decisively certain, though it is probable, that the splitting up of the trisaccharide raffinose into cane sugar and galactose, which was observed by Neuberg [4] with almond preparates, is due to melibiase. And no more certainly can a decision be made as to the identity of the almond enzyme with that of the bottom-fermentation yeasts.

The time-course of the hydrolysis of lactose as well as of raffinose by almond preparates was found by Willstätter and Csanyi to be mono-molecular,—they also observed a proportionality between enzyme-quantity and reaction-velocity. The divergent results communicated by Willstätter and Oppenheimer [5] in the cleavage of milk-sugar by Kaffir lactase are attributed by Kuhn [6] to enzyme destruction.

A preparative separation of galactosidases has not yet been carried out. According to Willstätter and Kuhn [7] the melibiase

[1] B. **35**, 3158 (1902).
[2] Proc. Roy. Soc. B. **80**, 321 (1908).
[3] H. **117**, 182 (1921).
[4] Bio. Z. **3**, 519 (1907).
[5] H. **118**, 168 (1921–22).
[6] In C. Oppenheimer, Die Fermente und ihre Wirkungen. 5. Aufl. Leipzig, 1925, p. 296.
[7] H. **115**, 180 (1921).

of yeast appears to resemble the cane-sugar splitting enzyme in its chemical behavior, since they are associated together even in extensively purified preparates.

2. POLYASES

a. Amylases

Occurrence.—The most important and most widely distributed enzymes which effect the transformation of the higher carbohydrates in animal and in plant organisms are the amylases or diastases. Their action relates to the degradation of starch and of glycogen, the degradation proceeding through the intermediate stages of the dextrins to maltose as an end-product. In the animal organism they are found chiefly in the digestive secretions, as in the saliva and in the pancreatic juice, and in the carbohydrate-storing organs such as the liver. In the plant world also they have been found mainly in those parts in which the storage and the mobilization of a reserve starch occurs, as in the seeds, in which the action of the enzyme appears during germination.

Measurement.—The various methods of determination recommended in the literature for the demonstration and the quantitative comprehension of the diastatic degradation of starch or glycogen may be classified from two points of view, according to whether they are based upon the alteration of definite properties of the substrate and the diminution of its quantity, or upon the measurement of the reaction products formed by their reducing action.[1] To the first class belong the processes of Pollak [2] and of Wolff and Fernbach [3] which follow the progressive dispersity alteration of starch solutions, their liquefaction, as well as also the much-used method of Wohlgemuth,[4] which is based on the alteration in the color reaction with iodine which occurs during starch decomposition. And finally there is the elegant newer method of determination employed by Rona and van Eweyk,[5] which determines nephelometrically the decrease in turbidity of glycogen solutions during the action of enzymes.

[1] For a complete résumé of these methods see Walton. "A Comprehensive Survey of Starch Chemistry," 1928, part 2, pp. 296-306.

[2] Wochenschr. f. Brauerei **20,** 595 (1903).

[3] C. r. **140,** 1403 (1905).

[4] Bio. Z. **9,** 1 (1910).

[5] Bio. Z. **149,** 174 (1924).

Those processes may be more reliable which determine the reducing action of the decomposed starch, e.g., with respect to Fehling's solution according to Bertrand [1] or with respect to hypoiodate according to the process of Willstätter and Schudel,[2] whose results were confirmed by Josephson.[3] For the applicability of these methods to the measurement of amylase action, which has been more precisely worked out by Sherman, Kendall, and Clark,[4] and by Willstätter, Waldschmidt-Leitz, and Hesse,[5] it is only necessary that the tested enzyme solutions contain no maltase. The comparative use of the various methods of determination for the measurement of starch decomposition, which sometimes lead to different results, has permitted important conclusions as to the nature of this process, as will be more fully explained later.

Salt Activation and pH Dependence.—The dependence of the action of animal amylase on the presence of neutral salts was first recognized through the observations of Nasse [6] upon saliva amylase and has been very extensively studied since that time. According to Bierry [7] animal and plant amylases differ in their capacity of activation by electrolytes; whereas according to the observations of Bierry and Giaja [8] pancreatic juice and pancreas extracts lose their diastatic action by dialysis and regain their activity upon the addition of salt, although malt amylase retains most of its effectiveness under the same circumstances and is not so spectacularly activated by salts. On the other hand, it appears from an investigation made by Haehn and Schweigart [9] that solutions of potato amylase behave similarly to those of the animal enzyme; by dialysis and ultrafiltration, it was possible to convert this enzyme into an inactive form, capable of activation by salts.

The theoretical significance of amylase activation by inorganic ions, in which a special influence is manifested by the anions and

[1] G. Bertrand and P. Thomas, Guide pour les manipulations de chimie biologique, Paris, 1910, p. 67.

[2] B. **51**, 780 (1918).

[3] B. **55**, 1758 (1923).

[4] Am. Soc. **32**, 1072 (1910).

[5] H. **126**, 143 (1922–23).

[6] Pflüg. Arch. **9**, 138 (1875).

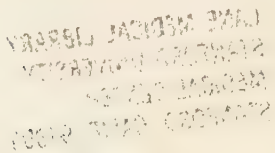
[7] Bio. Z. **40**, 357 (1912).

[8] C. r. **143**, 300 (1906).

[9] Bio. Z. **143**, 516 (1923).

in particular the Cl-ions, has already been treated in the general part of this book. It cannot as yet be definitely ascertained whether this influence concerns the dissociation of the enzyme, or whether it concerns the decomposition tendency of the substrate, as might be indicated in the investigations by Biedermann [1] regarding a salt hydrolysis of starch. It is true that an alteration of the *p*H dependence of saliva amylase and a displacement of the optimal hydrogen number according to the nature of the electrolytes employed have been observed in the researches of Ringer and van Trigt [2] as well as those of Michaelis and Pechstein,[3] and that these changes have been explained by the assumption of different amylase electrolyte complexes. This phenomenon, however, is to be regarded as a combined salt buffer activation in the sense of unequal capacities for activation with changing hydrogen number, according to Hahn and Harpuder [4] and according to Willstätter, Waldschmidt-Leitz and Hesse. The *p*H optimum of amylase from the pancreas has been found by Sherman, Thomas, and Baldwin [5] and by Willstätter, Waldschmidt-Leitz and Hesse to be independent of the quantity of Cl-ions present (corresponding to $pH = 6.8$), in accordance with the findings of Michaelis and Pechstein for saliva amylase and the observations of Holmbergh [6] for the liver enzyme. It appears that the different amylases of the animal organism, whose behaviorisms are largely coincident, are either identical or very closely related.[7] The optimal zone of action of most plant amylases, on the other hand, corresponds to a strongly acid reaction. Thus the *p*H optimum of malt amylase lies between $pH = 4.4$ and 5.2, according to various concordant observations made by Sherman and Schlesinger,[8] by Adler,[9] and by Lüers and Wasmund.[10] Similar

[1] Bio. Z. **129**, 282; **137**, 35 (1923).
[2] H. **82**, 484 (1912).
[3] Bio. Z. **59**, 77 (1914).
[4] Z. Biol. **71**, 287 (1920).
[5] Am. Soc. **41**, 231 (1918–19).
[6] H. **134**, 68 (1924).
[7] Compare however the remarkable new finding of H. Pringsheim and J. Leibowitz regarding a differentiation of ptyalin and pancreatic enzymes, B. **59**, 991 (1926).
[8] Am. Soc. **37**, 623, 643, 1305 (1915).
[9] Bio. Z. **77**, 146 (1916).
[10] Fermentf. **5**, 169 (1921–22).



values are reported by Sjöberg[1] as well as by Sherman, Thomas and Baldwin for the amylase of leaves and of *aspergillus oryzae*. Potato amylase, on the other hand, is closer in this respect to the animal enzymes, its optimal action being at pH = 6.7, according to McGuire and Falk.[2]

Kinetics.—The kinetics of starch hydrolysis has been studied a great deal. In the very first important description of amylase activity, and in fact of enzymic activity in general, Kirchhoff[3] recognized and stated that the starch is not wholly degraded to sugar, but that an uncrystallizable, degraded form of starch remains in the conversion liquor. Guerin-Varry,[4] a little later, reported that this gummy material is not saccharified even with an excess of the enzyme, but that after its isolation or separation it can be converted almost completely into sugar. Chittenden[5] and his co-workers also reported that the enzymic degradation of starch does not lead smoothly to the formation of the theoretical quantity of maltose, but that the reaction rather comes to a standstill considerably before this point is reached. In many other investigations,[6] motivated principally from a scientific brewing aspect, the attainment of a "resting stage" was described, with its corresponding "residual-, limit- or resistant-dextrin." According to more recent investigations by McGuigan,[7] by Euler and Svanberg,[8] and by Lüers and Wasmund, the saccharification limit or the "limited degradation" lies at the position of about three-quarters of the theoretical quantity of maltose. This phenomenon, in some cases, was formerly ascribed to an inhibiting influence of the cleavage products, especially as there was observed, in the manufacture of alcohol, a continuation of the saccharification, a "diastatic after-effect," following the fermentation of the maltose formed. Pringsheim and Fuchs[9] have shown mean-

[1] Bio. Z. **133**, 218, 294 (1923).

[2] J. Gen. Physiol. **2**, 215 (1920).

[3] Schweigger's Journal für Chemie und Physik, **14**, 389 (1815).

[4] C. r. **1**, 81 (1835).

[5] Trans. Conn. Acad., VI and VII (1885).

[6] See Walton. "A Comprehensive Survey of Starch Chemistry," New York. 1928.

[7] J. Biol. Chem. **39**, 273 (1919).

[8] H. **112**, 191 (1920–21).

[9] B. **56**, 1762 (1923); H. Pringsheim and K. Schmalz, Bio. Z. **142**, 108 (1923); H. Pringsheim and A. Reiser, Bio. Z. **148**, 336 (1924).

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

while that this subsequent saccharification depends upon the action of a complement contained in the yeast, which the amylase requires in order to complete the hydrolysis of certain starch degradation products, the so-called "limit dextrins." This suggests that many observations in the literature which report a characteristic difference in the saccharification limits by amylase from ungerminated and from germinated seeds,[1] e.g., from barley and malt, are to be explained by the formation of a further complement or activator, which appears during the germination. Differences in the reaction course of diastatic degradation are also manifested when comparing the two components of the starch granule, amylose and amylopectin. The observations which have been made with amylases of different origin agree that amylose is not only more rapidly but also more completely saccharified than the hull substance, amylopectin. Since the chemical structures of the varying components of the starch granule are so highly involved, it is extremely difficult to explain the intimate significance of the fact that characteristic differences are exhibited in the process of sugar formation by animal and plant amylases. It appears from the researches of Sherman and Baker,[2] as well as Willstätter, Waldschmidt-Leitz and Hesse, that the hydrolysis of starch by pancreatic amylase, up to a saccharification of about 40 per cent, follows a practically monomolecular course, in so far as we take as a basis for the calculation of the reaction constants the attainable limit of saccharification. On the other hand, Brown and Glendinning[3] first called attention to the fact that starch degradation by malt amylase usually proceeds more rapidly than that of an equation of the first order. Further, Lüers and Wasmund found, up to a transformation of 40 per cent, direct proportionality between the time of the reaction and the maltose formed. The findings of Sherman and Baker, also, in the case of Taka-diastase, indicate a considerable increase in the mono-molecular reaction constants.

Activity Scales.—The construction of definite formulas picturing the diastatic starch degradation will lack inherent justification so long as we fail to analyze this process into its partial processes and to comprehend these separately, especially since

[1] Confirmed by unpublished experiments of the author.

[2] Am. Soc. **38**, 1885 (1916).

[3] Soc. **81**, 388 (1902).

the homogeneity of amylase itself is not yet positively established. For the practical measurement of enzyme preparates, however, simple relations, as found for the first region of transformation by animal amylase, may be used as a basis, since there is found here likewise, according to the observations of Willstätter, Waldschmidt-Leitz and Hesse, an exact proportionality between reaction velocity and enzyme quantity. The same relation holds good also, according to Euler and Svanberg, and according to Lüers and Wasmund, for the comparison of the initial velocities in plant enzymes. Thus as a measure of pancreas amylase, Willstätter, Waldschmidt-Leitz and Hesse propose the " amylase unit (Am.E.) " as the 100-fold of that enzyme quantity for which, under the given conditions of experiment, the monomolecular reaction constant is found to be 0.01. And as a measure for the degree of purity, the " amylase value (Am.W.) " is used, which is given by the number of Am.E. in 1 cg. of the preparate. These expressions bear a simple relation to other definitions, given by Euler and Svanberg and by Sherman and Schlesinger [1] for the activity values of amylase preparates.

Liquefying and Saccharifying Actions of Amylase.—The divergent results which have been observed in the comparative application of different methods of determination for following starch degradations by amylases of varying origin, very early led to the hypothesis that diastatic starch hydrolysis was effected by two distinct enzymes. The distinction between two such enzymes was probably first pointed out by Maercker.[2] The possible existence of two distinct enzymes is still controversial. A special saccharifying enzyme, besides the starch-liquefying or depolymerizing enzyme, is assumed (according to the terms employed by Pringsheim,[3] an amylase and an amylobiase). In order to settle this problem a great deal of attention has been directed toward the efforts at characterization of the saccharifying and liquefying activity of various enzymes. Attempts have also been made to separate the two enzyme actions or to suppress one of them. Thus Pottevin [4] and more recently Ohlsson [5] announced that by a brief heating of amylase solutions it is possible to destroy entirely the saccharifying components of the enzymic system while yet

[1] As before.
[2] Chem. Centr., p. 559 (1878).
[3] B. **57**, 1581 (1924).
[4] Ann. Inst. Pasteur, **13**, 665 (1899).
[5] Soc. Biol. **87**, 1183 (1922).

retaining the liquefying action. Furthermore comparative experiments by Holmbergh [1] with liver amylase have shown that the presence of iodides inhibits sugar formation and, on the contrary, activates the liquefaction process. Hizume [2] sounds a discordant note in reporting that the addition of various electrolytes to solutions of pancreatic amylase causes no alteration in the relative activities. However, it has been chiefly the considerable differences in the liquefaction and saccharification capacities of the different types of amylase which have seemed to justify the theory of two enzymes occurring in varying proportions. Thus the researches of Sherman and Schlesinger [3] and of Kendall and Sherman [4] have demonstrated that with pancreatic amylase the liquefying action is predominant and that with malt amylase, on the contrary, the saccharifying action is predominant. Also, according to the observations of Effront [5] and of Chrzaszcz [6] liquefying activity is almost entirely lacking in resting seeds of cereals, while the saccharifying action is found to be normal; hence, a strong increase in liquefying capacity takes place upon germination. Meanwhile it is very noteworthy that a successful separation of the two postulated enzyme components by preparative methods has not been accomplished as yet. Thus it has been shown by the investigations of Sherman and Schlesinger with pancreas amylase, and of Lüers and Sellner [7] with malt amylase, who also used adsorptive methods, that the relation of enzymic actions—the liquefying and saccharifying—in all preparates is usually found constant, even though marked differences are found between the malt and pancreas enzymes themselves.

On the basis of this discovery it is not directly probable that more penetrating investigations will confirm the differentiation of the two diastatic enzymes. The divergencies manifested in the activity relations of the various amylases, which is most pronounced in the enzymes from barley and from malt, will be ascribed rather to the presence of special complements or activators, which are required for splitting up of certain definite atomic groups.

[1] H. **134**, 68 (1924).
[2] Bio. Z. **146**, 52 (1924).
[3] Am. Soc. **35**, 1784 (1913); **38**, 1885 (1916).
[4] Am. Soc. **32**, 1087 (1910).
[5] C. r. **174**, 18 (1922).
[6] Bio. Z. **142**, 471 (1923).
[7] Wochenschr. f. Brauerei, **42**, 97, 103, 110 (1925).

Alpha and Beta Amylases.—Worthy of special mention are the results recently published by Kuhn [1] regarding the action mechanism of the various amylases. These have led to the differentiation of two groups of diastatic enzymes, the α-amylases and the β-amylases, which attack different points of the starch molecule. Optical analysis of the sugars formed upon rapid degradation of starch, whose directions of muta-rotation were found to be different, has led to the discovery that the α-amylases produce an α-maltose as the end product of starch degradation, whereas, the action of β-amylases results in the primary formation of β-maltose. Pancreas and Taka-diastases can be considered as the most important members of the α-amylase group, while malt amylase can be considered as a characteristic member of the β-amylase group. Important deductions as to the structure of the starch molecule have been based upon these observations. Thus, it is assumed that alternating α- and β-glucosidic linkages and non-furoid grape sugar residues are existent in the starch molecule.[2]

Purification.—The most pronounced advance in the preparative isolation of diastatic enzymes has been obtained in the case of animal amylases. According to the principle first followed by Danilewsky [3] for separating the enzyme of the pancreatic juice, Cohnheim [4] purified saliva amylase by adsorption on calcium phosphate and elution with water and reported that the preparate was free from protein. He also made the important observation during the comparison of the adsorptive behavior of the pancreatic enzymes that " the starch converting ferment attaches itself much less intimately to minute particles than does the fibrin-dissolving ferment." However, later researches, devoted to the purification of animal amylase, do not support entirely these important earlier experiments on the adsorptive behavior of the enzyme. Thus the preparative examples presented by Sherman and Schlesinger [5] for the isolation of pancreas amylase have been confined to the

[1] A. **444**, 1 (1925).

[2] The new finding of H. Pringsheim is in agreement with this assumption. In this, the direct scission of starch to grape sugar by the combined action of α- and β-amylases has been observed. B. **58**, 1262 (1925); **59**, 991, 996 (1926).

[3] Virch. Arch. **25**, 279 (1862).

[4] Virch. Arch. **28**, 241 (1863).

[5] Am. Soc. **37**, 1305 (1915).

concentration of the enzyme from commercial gland preparates by fractional precipitation of the extract at a low temperature. By this process, which occasions much loss, it has not been possible, even in the best preparates, whose amylase value was about 6.1, to separate the amylase either from trypsin or from protein-like substances.

Willstätter, Waldschmidt-Leitz and Hesse [1] first reattempted the purification of pancreas amylase by means of adsorptive methods. It was found thereby that, among the three most important pancreatic enzymes, amylase exhibits the least pronounced adsorptive affinities. It is indifferent towards electropositive and electronegative adsorbents, whereas lipase is taken up unspecifically and very easily by the adsorptive agents studied, and trypsin, on the other hand, is taken up chiefly by acid adsorbents. Hence the three pancreatic enzymes can be separated from each other by reason of their different adsorptive behavior. From the mixture of the three enzymes as it occurs for example in the glycerine extracts of the dried pancreas, the lipase is first removed by adsorption with alumina in an acid reaction, then trypsin is taken up from the residuary adsorption solution by means of kaolin. Whereas, the loss of amylase, conditioned by a partial adsorption of the enzyme under the influence of coadsorbents, is not very great during this process.

Since amylase is lacking in adsorption affinities, it has not been possible as yet to increase its enzymic concentration by such methods to so great an extent as in the case of the other enzymes. Still, when the enzyme has reached a state of enzymic homogeneity it exhibits a certain degree of adsorptive capacity due to its concomitant substances, and this may be utilized to attain a further degree of purification. At this degree of purity it is taken up by alumina in a neutral reaction and most appropriately with a high alcoholic content, which, as in the case of other amphoteric substances such as amino acids or peptides, causes the development of the acidic properties in the coadsorbents. In the alkaline elutions of the alumina adsorbates, the amylase, free from protein but still containing carbohydrate, is obtained in an appreciably increased purity-degree, corresponding to the amylase value 64, about 125 times as concentrated as in the dried gland.[2]

[1] H. **126**, 143 (1922–23); **142**, 14 (1924–25).

[2] Translator's Note.—See the critique of H. C. Sherman, M. L. Cald-

The adsorbability of amylase by alumina, which has been observed under certain conditions and which may be employed for its purification, is based, however, merely upon an association of the enzyme with concomitant substances. It has been found possible to so purify the enzyme by pre-adsorption, i.e., by adsorption of a part of the substance, that it can no longer be taken up by alumina either from the aqueous or from an alcoholic solution.[1] Its true adsorptive behavior therefore becomes recognizable only in purer solutions. In this condition it is possible to accomplish the adsorption of amylase by the use of much stronger basic adsorbents, i.e., beryllium or ferric hydroxides. However, it has not yet been investigated as to whether concomitant substances might not be responsible for even this behavior.

Purification of Malt Amylase.—Among the plant amylases, the enzyme of germinated barley has been most thoroughly studied from a preparative standpoint. The much advanced assumption that the enzyme is, in the main, first formed from a pre-stage during the germination of barley and with the cooperation of oxygen, and also that it is formed, according to Lüers [2] as well as Baker and Hulton,[3] from protein substances of the barley grain, does not appear to be correct, according to recent investigations by Syniewski as well as according to unpublished experiments of the present author. The earlier observations, whose analytical principles are inadequate, are to be understood

well and M. Adams [Am. Soc. **48**, 2947 (1926)] in which it is pointed out that the above value is obtained by determining the activity of the alkaline elutions of the alumina adsorbates and then interposing a prolonged period of dialysis against running water before determining the solids for the calculation of enzymic value. They maintain that the enzyme in this solution, containing glycerol, is appreciably dialysed away during the process. Correspondingly the resulting calculated amylase value would be considerable higher than the true value. Repetition of this procedure by Sherman and co-workers under the most favorable conditions which they were able to establish yielded a preparate the activity of which was about half what they had obtained previously by precipitation methods. [Am. Soc. **33**, 1195 (1911); **34**, 1104 (1912)]. Sherman and co-workers for a number of years have postulated the protein nature of the amylases and in that their purest preparates here obtained have shown protein reactions their position is still maintained.

[1] R. Willstätter, E. Waldschmidt-Leitz, and A. R. F. Hesse, H. **142**, 14 (1924–25); as well as still unpublished experiments of the author.

[2] Bio. Z. **104**, 30 and particularly p. 71 (1920).

[3] Soc. **121**, 1929 (1922).

as applying not to a quantitative but to a qualitative difference of the amylases in barley and malt. The field of action, measured by the formation of reducing sugar, appears to be enlarged by the germination.

The attempts in regard to the concentration of malt amylase found in the earlier literature confined themselves essentially to the use of fractional precipitation by the organic solvents alcohol or acetone, from aqueous or aqueous-alcoholic malt extracts, according to the example of Lintner.[1] According to Sherman and Schlesinger [2] the enzyme was extracted from the malt with dilute alcohol and, after dialysis of the extract, was precipitated by organic reagents or by salting out with ammonium sulfate. The best preparate obtained by these investigators corresponded to an amylase value of about 2.8. Also by the use of adsorption media, e.g., of alumina, Lüers and Sellner [3] were able to increase the enzymic concentration only to an amylase value of about 1.6. Fricke and Kaja [4] subjected solutions of commercial amylase preparations to electro-dialysis and electro-osmosis; the resulting preparates were reported as being protein-free, but yet containing considerable quantities of carbohydrates. The concentration of the enzyme, which was raised to about 6-fold by these operations, cannot be safely compared with that attained with other preparates in the literature.

A preparative purification of the enzyme from *aspergillus oryzae* has also been carried out by Sherman and Tanberg.[5] The purer preparates, which were obtained by precipitation with ammonium sulfate and by fractional alcohol precipitation and the enzymic concentration of which was about 30-fold that measured in the commercial preparates, still contained appreciable quantities of protein substance.

b. Inulase, Lichenase, Cellulase

To the group of polyases belong, besides the starch and glycogen splitting enzymes, also a number of other representatives,

[1] J. pr. **34**, 378; **36**, 481 (1886–87).
[2] Am. Soc. **37**, 643 (1915).
[3] Wochenschr. f. Brauerei **42**, 97, 103, 110 (1925).
[4] B. **57**, 310, 765 (1924).
[5] Am. Soc. **38**, 1638 (1916).

some of which are capable of hydrolysing reserve carbohydrates and some the true plant framework substance. However, their modes of action and properties have not been so thoroughly investigated.

Among these may be mentioned inulase, which hydrolyses the reserve substance, inulin, in subterraneous tubers and roots, to d-fructose. The specific actions and the properties of the enzymes, which are found, for example, in mold fungi like *aspergillus niger* and *penicillium glaucum*, and which were recently described by Pringsheim and Kohn,[1] are not very intimately understood, especially since they have not yet been separated from the associated saccharase.

Also lichenase, which effects the cleavage of the lichen-starch, lichenin, to cellobiose, may be mentioned here. This has been found and more closely described in malt extracts by Pringsheim and Seifert [2] and also in the intestinal tract of the snail by Karrer, Joos and Staub.[3] This enzyme is to be classified with the hemicellulases, whose actions have been observed, it is true, in the germination of seeds with the dissolving of the hemicelluloses, but whose individuality has not been more closely characterized.

The same holds for the true cellulose-degrading enzyme, whose action has been assumed in the activity of wood-destroying bacteria and fungi. Pringsheim has isolated a cellulase from cellulose fermenting bacteria and described its action. Boynton and Millar [4] likewise have demonstrated the presence of a cellulase in the digestive tract of the ship worm. These marine wood-borers are well known for their destructive action on wharf piling and other timbers exposed in sea water. The extraction of the cellulase, in this later case, was carried out under conditions which would exclude the presence of bacteria. Karrer [5] using a cellulase from snails, has studied the action on various types of native cellulose and viscose. In the case of the viscose, the degree of hydrolysis appears to bear a relation to the conditions under which it is precipitated.

[1] H. **133**, 80 (1924).

[2] H. **128**, 284 (1923); H. Pringsheim and J. Leibowitz, H. **131**, 262 (1923) H. Pringsheim and W. Kusenack, H. **137**, 264 (1924).

[3] Helv. **6**, 800 (1923); **7**, 518 (1924).

[4] J. Biol. Chem. **75**, 613 (1927).

[5] Helv. **8**, 797 (1925); **9**, 893 (1926); **10**, 430 (1927).

V. CATALASES

Characteristics and Significance in Respiration.—The decomposition of hydrogen peroxide by living plant and animal tissues was observed at a comparatively early date, but it was not until some time later that the phenomenon was first attributed, by Loew,[1] to the action of a special enzyme. This enzyme was termed catalase and its extraordinarily wide distribution in nearly all the tissues and secretions of plant and animal organisms was soon recognized. The only known action of the enzyme is the decomposition of hydrogen peroxide into oxygen and water, and it is not capable of decomposing other peroxides, such as the organic derivitives of hydrogen peroxide. Further, the oxygen liberated appears to be in the molecular inactive form.

The wide distribution of catalase in organisms suggests that this enzyme must possess an important physiological function in the facilitation of biological oxidation processes, even though its substrate, hydrogen peroxide, is not observed in living tissue. Thus, a series of noteworthy views regarding the part played by catalase in the respiration of the tissues has been published. These are based principally upon the common assumption that the task of the enzyme is to remove the cell poison, hydrogen peroxide, formed as an intermediary in oxidation processes. Thus, Wieland,[2] who regards most oxidation processes, even biological oxidations, as dehydrogenation reactions, has advanced the theory that the respiration enzyme is able to carry the hydrogenation of molecular oxygen only to the stage of its semi-saturation with hydrogen, which corresponds to hydrogen peroxide. At this point the action of the catalase sets in, which together with the removal of this cell-toxin simultaneously provides fresh oxygen for the dehydrogenation. The discovery that only oxygen-requiring cells contain catalase, and that anaerobic cells on

[1] Bull. Dept. Agri., Washington, **68**, (1900); see also Bio. Z. **34**, 354 (1911).

[2] B. **55**, 3639 (1922); Ergebn. d. Physiol. **20**, 477 (1922).

the contrary do not contain catalase, and that the distribution of the catalase is related to the oxygen-requirements of the various cells, at any rate justifies the assumption that these enzymes appear to be indispensable for the conduct of the oxidation process. This would be true whether we describe the process, with Wieland, as dehydrogenation reactions, in the sense of a hydrogen activation in the substrates of the oxidation, or whether we regard it, with Bach and Chodat,[1] as direct oxidations, involving the operation of activated oxygen.

For the determination of catalase action, two equally valuable methods of measurement are at our disposal. We can use either the volumetric measurement of the oxygen evolved or else the titration of the undecomposed hydrogen peroxide with potassium permanganate.

Hydrogen-Ion Dependence.—The *p*H dependence of catalase action was first exactly determined with the enzyme from liver by Sörensen;[2] the optimum of enzyme action was found to be at *p*H = 7.0, corresponding accordingly to a neutral reaction. This result was confirmed by later researches of Michaelis and Pechstein,[3] by Morgulis[4] as well as by Rona and Damboviceanu,[5] and further amplified by the finding that the breadth of the optimal zone of action and the position of the activity-*p*H curve depend upon the content of neutral salts in the solution. With increasing salt content the dependence on the hydrogen number is intensified, while at the same time the maximal attainable reaction velocity is decreased.

Kinetics.—The observations of the literature upon the kinetics of hydrogen peroxide decomposition by catalase manifest noteworthy differences between the action of the plant and the animal enzymes, differences that may be ascribed in part to a difference of stability of the enzyme material. Thus the experiments of Issajew[6] with catalase solutions from dried yeast led to the result that the transformation of hydrogen peroxide by the enzyme may be described by an equation of the first order, with the use

[1] Soc. Biol. **83**, 1544 (1920); Arch. int. de Physiol. **18**, 403 (1921).
[2] Bio. Z. **21**, 131, 279 (1909).
[3] Bio. Z. **53**, 320 (1913).
[4] J. Biol. Chem. **47**, 341 (1921).
[5] Bio. Z. **134**, 20 (1922).
[6] H. **42**, 102 (1904); **44**, 546 (1905).

of dilute hydrogen peroxide solutions at 25° or with higher substrate concentrations at 0°. The results obtained by Euler [1] with the catalase from fungus extracts are similar, giving a direct proportionality between enzyme quantity and reaction velocity.

These relations have been observed for the action of animal catalase only under definite conditions, under which no destruction of the enzyme takes place, namely, at a low temperature and by the use of very dilute hydrogen peroxide. Thus a monomolecular course of the reaction and proportionality of the catalase quantity and reaction constant have been described by Senter [2] for blood catalase and by Hennichs [3] for liver catalase. On the other hand Michaelis and Pechstein, Morgulis and Rona and Damboviceanu have reported that concentrated catalase solutions from an animal origin possess a relatively stronger activity than dilute solutions of the enzyme. According to Morgulis the kinetics of catalase action is conditioned by the existing quantity relation of the enzyme and substrate. The reaction course of hydrogen peroxide decomposition, which can only be expressed by an equation of the first order in the action of larger enzyme quantities, is found to be relatively slower with diluted enzyme solutions and corresponds to a bi-molecular reaction equation under certain conditions; this is to be explained by the marked instability of dilute catalase solutions and by an enzyme-destroying as well as an inhibiting action of the hydrogen peroxide.

In agreement with this is the fact that, in the investigations described, equal transformations were not observed in given time periods, these two periods being inversely proportional to the enzyme quantities used. On the other hand, according to Morgulis and to Rona and Damboviceanu, the hydrogen peroxide quantities decomposed in equal periods of time are directly proportional to the catalase quantities. It is to be readily seen that from such observations the conclusions would be derived that the catalase is not capable of acting as a true catalyzer, but that a given catalase quantity is capable of transforming only a given quantity of hydrogen peroxide.

Catalytic Nature and Units of Measurement.—According to such a theory, the enzyme itself is supposed to undergo an oxida-

[1] Hofm. Beitr. **7**, 1 (1905).
[2] Ph. Ch. **44**, 257 (1903); **51**, 673 (1905).
[3] Bio. Z. **145**, 286 (1923–24).

tion in the sense of a coupling reaction at that point in its molecule, which, as its specifically active group, effects the transformation of the hydrogen peroxide. The amount of this oxidation is governed by the given conditions of the experiment such as temperature and concentration of the hydrogen peroxide. The results obtained in the refutation of similar views upon the mode of action of peroxydasic enzymes should emphasize the necessity for caution in this regard. Taking into consideration the other sorts of observations upon plant catalase, it is far more probable that the animal enzyme also is to be regarded as a true catalyzer, whose activity suffers no injury in the course of the reaction itself, and whose destruction, which seems to take place more readily than in the case of the plant enzyme, occurs independently of the true enzymic reaction. Hence we are justified in basing the measurement of the activity of catalase preparates upon the observed mono-molecular reaction constants. Thus we use the "catalase-value (K.W.)" as a measure for the cencentration of the enzyme, i.e., the reaction constant obtained for the action of 1 g. of the preparate upon 0.02 n H_2O_2 at 0° and the optimal hydrogen number.

The theoretically important analogy between the action of catalase and that of inorganic catalyzers, to which Faraday has already called attention, seems to be of special interest here since the effectiveness of inorganic catalyzers is much exceeded quantitatively by that of enzymic materials. This fact is evident from Table XXXVII, which compares the activity of different catalase preparates of the literature with that of colloidal platinum.

TABLE XXXVII

Activity of Catalase and Inorganic Catalysts

Preparate	K.W.	Observer
Colloidal Platinum	1.8	G. Bredig
Blood Catalase	35.0	A. Madinaveitia
Liver Catalase	67.0	F. Batelli
Liver Catalase	532.0	S. Hennichs

Preparation.—The older processes for the preparative isolation of catalase from plant or animal material confine themselves

essentially to the use of alcoholic precipitations in the enzyme extracts, as has been described by Batelli and Stern [1] when preparing the liver enzyme. The increase in the enzymic degree of purity thus obtained was not considerable, even though Euler [2] obtained in this way a separation of the starch-splitting enzyme in malt extracts, and even though Senter [3] made the analytically and theoretically important discovery that in this manner the blood catalase, " hämase," can be obtained free from iron. The methods of a selective adsorption of catalase from blood were first carried out by Willstätter and Madinaveitia [4] on the basis of the observation that the enzyme is taken up from its solutions very readily by fat, in accordance with the increased catalase content of fat-containing tissues. But the low stability of the enzyme in the fat adsorbates, especially in attempts at its liberation, adversely affected the success of the procedure. The best preparate obtained corresponded only to the catalase-value of 35.

The newer results by Tsuchihashi [5] appear to be of considerable importance to the characterization of blood catalase. In this work the enzyme is supposedly freed from protein substance by adsorption on tri-calcium phosphate and by subsequent elution with secondary phosphate solution. According to this, the view expressed by Waentig and Steche [6] regarding the protein nature of catalase, derived from its instability with respect to trypsin, is not sustained. The most important preparative progress has been made by Hennichs [7] with the systematic use of the adsorptive agents, alumina and kaolin, for the purification of liver catalase. By adsorption of the enzyme with kaolin in acid reaction and dissolving out by means of weak alkaline phosphate, preparates of the catalase-value 532 were obtained. And by repeated adsorption of the catalase on alumina and dialysis of the alkaline elutions (although this resulted in a great loss in enzymic activity) preparates of similar concentration were obtained

[1] Soc. Biol. **57**, 375 (1905).

[2] Hofm. Beitr. **7**, 1 (1906).

[3] Ph. Ch. **44**, 257 (1903); **51**, 673 (1905).

[4] A. Madinaveitia, Zur Kenntnis der Katalase. Promotionsarbeit Zurich 1912.

[5] Bio. Z. **140**, 63 (1923).

[6] H. **83**, 315 (1913); **93**, 228 (1914–15).

[7] Bio. Z. **145**, 286 (1924).

(K.W. 521), corresponding to a concentration increase of about 16 times that of the source material.[1]

It is to be expected that a more selective process of adsorption, modeled after this example, will permit the attainment of a further considerable increase of the purity-degree, although the success of the preparative purification of catalase may be adversely affected by the strongly increasing instability of the enzyme; this must be combated by the use of stabilizers.

[1] Adsorption with kaolin has been carried still further (K.W. 1250) in more recent experiments by S. Hennichs. It is of importance that in these purifications, no proportionality between the enzymic action and the iron content of the preparate was observed [B. **59**, 218 (1926)].

VI. PEROXYDASES

Distribution and Function.—The peroxydases, whose actions consist in the transfer of peroxydasically linked oxygen to oxidizable substances, are widely distributed as auxiliary enzymes for the facilitation of biological oxidations. They are found, for example, in particularly plentiful quantities in the roots and the seedling sprouts of the higher plant organisms. These enzymes are to be sharply differentiated from the catalases by their specific modes of action, particularly by the fact that they do not decompose hydrogen peroxide in the absence of oxidizable substances, as is the case with the catalases. In fact, they occasion only an activation of the peroxides. Furthermore, the apparently contradictory observations of Abel [1] referring to the "catalase-action" of plant peroxidase in the presence of iodine ions are not to be ascribed to a participation of the enzyme in the true oxygen-liberating reaction; the influence of the enzyme is confined rather to only a definite partial process of the reaction, namely, to the oxidation of the hydriodic acid to iodine.

Procedures and Scales for Measurement.—For the detection and determination of peroxydasic enzymes, the oxidation of organic dye bases is ordinarily used, or else phenols in the presence of hydrogen peroxide. Thus the action of the peroxydase is determined by the formation of malachite green from its leuco-compound, the quantity of the dyestuff produced being ascertained colorimetrically. Or it can be determined by the much used process of oxidizing pyrogallol to purpurogallin, an orange-red, ether-soluble dyestuff, the constitution of which has been elucidated by Willstätter and Heiss.[2] The quantity of the purpurogallin is determined either colorimetrically or by weighing the ether extracted product. An exact procedure for measuring the peroxydase quantities, however, was first worked out by Willstätter and Stoll,[3] who demonstrated for the first time the importance of a

[1] Z. El. Ch. **28**, 489 (1922). [2] A. **433**, 17 (1923). [3] A. **416**, 21 (1917–18).

reliable method of determination as a prerequisite for preparative work.

The earlier theories of Bach and Chodat [1] that a definite relation existed between the substances that have part in the reaction, peroxydase, hydrogen peroxide and purpurogallin, and that the enzyme as well as hydrogen peroxide is used up in the oxidation process, have proved to be erroneous. This resulted because, in order to produce unlimited quantities of the oxidation product with a given quantity of the enzyme, it was only necessary to determine and maintain those conditions under which the enzyme would be perfectly preserved in its action—accomplished by avoiding the acid reaction and by using very dilute solutions of hydrogen peroxide. At higher concentrations of peroxide, according to Willstätter and Weber,[2] the peroxydase is not destroyed but considerably inhibited.

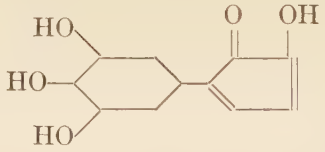
The novelty of this phenomenon is particularly demonstrated by the fact that the reaction may come to a standstill when there are still substantial quantities of peroxide and substrate remaining in the reaction mixture. This suppression proceeds the more rapidly the higher the concentration of peroxide. It is positively demonstrated, however, that the phenomenon is not due to the peroxydase being consumed in the reaction or being destroyed by the higher concentrations of peroxides. Experiments show that the reaction can again be initiated either by increasing the concentration of the substrate or by removing some of the peroxide. For instance, by the action of catalase, the peroxide may be reduced to such concentration that the reaction will proceed once more with undiminished intensity. A perfectly uniform reaction course can be obtained by dropping in peroxide so slowly that its concentration always remains very low. For the practical measurement of peroxydase this is not necessary as it is shown that the usual methods are sufficiently reliable at very low concentrations of peroxide. At 0.25 mg. peroxide in 100 ccm., for instance, the quantity of malachite green formed is directly proportional to the quantity of enzyme and the duration of the test.

Accordingly, the measures established for the quantity and concentration of the peroxydase are based on the fact that, under the conditions of the determination, the activity of the enzyme undergoes no alteration and hence that equal transformations are

[1] B. **37**, 1342, 2434, 3785 (1904). [2] A. **449**, 175 (1926).

obtained in given periods of time which are inversely proportional to the quantities of peroxidase employed. Thus Willstätter and Stoll express the purity degree of the enzyme preparates by the "purpurogallin number (P.Z.)," namely, the quantity of purpurogallin in milligrams formed by 1 mg. of the preparate acting on 5 g. of pyrogallol and 50 mg. hydrogen peroxide in 2 liters water at 20° and for 5 minutes. Upon this definition is based further the measure for the quantity of peroxidase, the "peroxidase unit (P.E.)" which is represented by 1 g. of substance of purpurogallin number 1, or by 1 mg. of substance of purpurogallin number 1,000. The number of peroxidase units present is obtained by the product of the purpurogallin number and the quantity of the preparate.

Reliability of the Purpurogallin Method.—Because of certain unusual and rather unexpected variations in the activity of peroxidase preparates (an increase of activity on standing, which is to be taken up later), some apprehension has been manifested as to the reliability of the purpurogallin method. For one thing the oxidation reaction is by no means a simple one. It proceeds by condensation of two molecules of dehydrogenated pyrogallol, with successive opening of the benzol nucleus and closure to the cyclopentane derivative, purpurogallin,



In order to disprove the possible combination of the enzyme with one of the intermediate products, comparative tests were made by means of an oxidation with leuco malachite green. This is a simple oxidation reaction involving the cleavage of only 2 atoms of hydrogen or the taking up of 1 atom of oxygen. Its oxidation under the influence of peroxydase was first noted by Adler.[1] The conditions by which accurately reproduceable results might be obtained were first worked out by Willstätter and Weber.[2] Certain low and fairly definite quantities of peroxide must be used and the leuco base must be purified by recrystallizations from ligroin and alcohol. Under the conditions developed,

[1] H. **41**, 58 (1904). [2] A. **449**, 156 (1926).

it was found that the results were in close agreement with those obtained by oxidation to purpurogallin and, further, coincided in those cases where a supposed abnormality of peroxydase content occurred. Accordingly, the complete validity of the purpurogallin method is considered as established. The method is used as a control in the researches of Willstätter on the preparative purification of peroxidase.

Preparative Procedures.—The enzymes from the horse-radish root and the turnip were used primarily in the preparative works of Willstätter. Three procedures were involved in obtaining crude, enzyme-rich solutions from the comminuted plant root: dialysis in the plant material, next precipitation of the enzyme with oxalic acid, and, finally, fractional extraction with alkali.

Dialysis of the Plant Material.—The first step of the preparative elaboration, dialysis of the coarsely fragmented plant substance, is based upon the experience that the enzyme in this operation is held back to a large degree by the intact cells, while easily diffusible, low-molecular, concomitant substances are removed. The losses in enzymic substance which occur during this process concern only a fraction, at most one-fifth, of the total enzyme content; they are attributed to the mechanical destruction of the cell-structure upon comminution. Rather, it has been found that, after the initial migration of a small quantity of peroxidase, a new formation of the enzyme takes place and to a very appreciable extent. This is expressed in the increase of the peroxidase value and is due to the continuation of vital activity in the plant cells during dialysis. Only after a very prolonged dialysis—of several weeks—is a retrogression of the peroxidase content to be observed once more, which is due this time to the exosmosis of the enzyme becoming greater than its formation. There have been numerous observations which would seem to indicate the existence of two different kinds of peroxidase in the plant-cell, a soluble and an insoluble form, e.g., the behavior during dialysis.[1] Willstätter and Pollinger[2] have attributed this to the phenomenon that a part of the enzyme, dissolved in the cell-sap, easily escaped from the cell, while another part, bound to the protoplasm, can be liberated only after complete destruction of the cell-structure. Thus the distinctions between the soluble and the insoluble

[1] R. Willstätter and K. Riehmann, A. **422**, 47 (1920–21).

[2] A. **430**, 269 and particularly p. 312 (1922–23).

enzyme are removed by the discovery that it is possible, e.g., by dilute alkali, to carry over the entire amount of the peroxidase into the solution.

Precipitation with Oxalic Acid.—The second stage of the purification depends upon the observation that the action of oxalic acid serves to fix the peroxidase more firmly upon the plant substance, while the dialysis at the same time progresses without diminution. The alteration of the cells, which results from the treatment with acid and which initiates the activity of autolytic cell enzymes, leads to the transformation of a series of high-molecular substances of the cell-content into more easily diffusible, low-molecular substances. On the other hand, the peroxidase, by the action of acid, is so precipitated upon coagulated plant substance that the material, upon termination of the dialysis, can be comminuted and pressed out without any considerable loss of the enzyme.

Fractional Extraction.—Extraction of the peroxidasic enzymes from the root pulp is next accomplished by the third step of the isolation, that is, complete neutralization of the acid contents. Thus by gradual action of dilute alkali, baryta for example, a fractional removal of the plant material is effected, in which the enzyme goes over into the weakly alkaline extract. The extract is subsequently concentrated in vacuo and the enzyme separated by fractional precipitation with alcohol, according to the process of Bach and Chodat. The described process of isolation leads to peroxidase preparates of considerably increased purity-degree, corresponding to P.Z. 100 to 260 from horse radish, or P.Z. 200 to 350 from the turnip, with an average enzyme yield of about one-third.

Precipitation with Mercury Salts and Tannic Acid.—A further purification of the enzyme from these crude preparates was attained by Willstätter and Stoll upon observing that a part of the glucosidic substances accompanying the peroxidase could be separated out by precipitation with mercury salt, while the enzyme remained behind in the portion not precipitable by the heavy metal. The enzymic concentration of the substance, freed from mercury by precipitation with alcohol rose considerably hereby, in the best example to P.Z. 670. However, the action of mercury salt for the purification of peroxidase appears to be but difficultly applicable in the preparative method and the results are not easily reproduceable. For the analytical characterization of the enzyme,

the method is surpassed by precipitation of the peroxidase itself with tannic acid, which Willstätter and Pollinger have employed for the isolation of the enzyme, either from its crude solutions or from the solutions pre-purified by adsorption. This reaction, whose analytical importance depends chiefly on the elimination of tenaciously associated, iron-containing impurities, appears to be a function of the enzyme itself—it is observed, namely, in the crude preparates of the peroxidase as well as in the purest preparates thus far obtained. Its preparative evaluation is rendered difficult, however, by the insolubility of the tannin precipitate, the decomposition of which is effected only after its preparation from pre-purified enzyme solutions. Thus the process for purifying high-value peroxidase preparates by means of tannin consists in the decomposition of the precipitate, dissolved in acetic acid and alcohol, by means of kaolin, from which the adsorbed enzyme, now free from tannin, may be dissolved out by the action of alkali.

Adsorption by Alumina and Kaolin.—The application of adsorption methods to the purification of peroxidase has proved to be more important than even the elaboration of precipitation methods. Thus the adsorption behavior of plant peroxidase toward alumina is very characteristic and has been successfully used for its purification. The discovery that the peroxidase is taken up only very slightly from the aqueous solution of alumina permits a certain pre-purification of the solutions by pre-treatment with the adsorbent, which accomplishes a separation of a part of the admixtures without enzyme loss. Experiments have been based upon the further observation that the enzyme is adsorbed by alumina after the addition of alcohol. Its distribution between solution and adsorbent is governed by the influence of solvents upon its attachment and liberation from the adsorbent.

The phenomenon, that peroxidase is taken up by alumina only from an alcoholic solution, corresponds to the predominantly basic properties of the enzyme. It recalls the behavior of other amphoteric substances, e.g., that of the polypeptides and amino acids, whose acid functions are manifested only in alcoholic solution. Likewise, in the course of purification no alteration is to be observed in the qualitative adsorption behavior of the peroxidase. The preparative process for adsorption of the enzyme on alumina, furnished by this discovery, consists in taking it up by the adsor-

bent from aqueous-alcoholic solutions, and in liberating it from the adsorbate, most suitably by the action of water containing carbon dioxide.

The basic properties of peroxidase are further exhibited by its behavior toward kaolin, by which it is readily and completely taken up. The addition of alcohol can be dispensed with here. For preparative application the most favorable conditions are found when adsorbing the enzyme from very dilute acetic acid solutions, while elution of the kaolin adsorbate is effected by the action of dilute ammonia. These operations can frequently be carried out without loss of enzymic activity, particularly when purer enzyme preparates are used. For separating the peroxidase from concomitant substances, as in the case of other enzymes, a great advantage is provided by changing the polarity of the adsorbent. Thus by means of kaolin, success was first attained in eliminating the obstinately attached glucosidic impurities, which had falsified the composition of older preparates.[1] In elutions of the kaolin adsorbates, the peroxidase of the plant roots was obtained free from carbohydrate-containing substances. And in another example also, in the elaboration of cereal seedling sprouts, the use of kaolin adsorption has made it possible to isolate the enzyme directly in a high degree of purity. Finally, the preparative importance of kaolin adsorption is exemplified in the decomposition of the peroxidase-tannin compound, to which reference has already been made.

Spontaneous Increase of Peroxidase Activity.—The use of adsorptive methods in the investigation of Willstätter and Pollinger has also been of help in explaining a phenomenon which is very frequently observed in the course of the preparative purification of peroxidase, namely, an increase of the peroxidasic action. This phenomenon, which seems to render uncertain the quantitative determination of the enzyme as a basic principle for preparative work, is encountered, e.g., upon storing peroxidase solutions or solid peroxidase preparates, and manifests itself by a pronounced gain in enzymic activity. It is further encountered in adsorption processes, e.g., with alumina, upon concentrating dilute enzyme solutions under diminished pressure, or finally, in the isolation

[1] See R. Willstätter and A. Stoll, A. **416**, 21 and particularly p. 56 (1917–18); R. Willstätter and A. Pollinger, A. **430**, 269 and particularly p. 278 (1922–23).

of peroxidase from its solutions by precipitation with alcohol. Willstätter and Pollinger seek to explain this discovery by the assumption that a decomposition product of peroxidase, and moreover one very closely related to the enzyme itself, is capable of inhibiting the active group by association with the enzyme; further, this inhibition is eliminated by separation of the material or again by voluntary decomposition of the material. It was, in fact, found possible to separate these decomposition products by adsorption on alumina from enzymes which had suffered a loss in enzymic concentration through decomposition, and thereby to restore the enzyme to its original degree of purity.

Increase in Purity-Degree.—By the combined application of adsorption and precipitation methods, Willstätter and Pollinger obtained a peroxidase of a high degree of purity. Thus in an example of the preparative elaboration of the enzyme from turnips a preparate with the purpurogallin number 3160 was obtained by successive adsorptions with kaolin and alumina, precipitation of the enzyme with tannin, decomposition of the tannin precipitate with kaolin and subsequent elution. And in another example the purity-degree was raised much higher by adsorbing 5 times on alumina and a single time on kaolin; the purpurogallin number in the dissolved enzymic substance was observed to be 4900, corresponding to an almost 20,000 times greater concentration than that of the dried root.

Relation of Color and Activity.—The position so far attained in preparative technique has furnished important new information with regard to the analytical characterization of the enzyme. Thus the investigation of purer peroxidase preparates has permitted the recognition of a characteristic property of the enzyme, namely, its color. Peroxidase in solution exhibits a clear, brownish-red color, which recalls certain porphyrines obtained from the blood and leaf dyestuffs, particularly pyroporphyrine. The certain recognition of the relations between peroxidase action and color are complicated by the circumstance that the enzyme is associated with non-enzymic, concomitant substances of similar color in the crude solutions and that the decomposition products of the enzyme are themselves colored. Thus it has been shown that the color persists after destruction of the purer peroxidase by acid or by heat or even by its spontaneous decomposition.

These observations correspond to the information obtained on

the relations of peroxidasic action and color in the course of preparative purification. Thus it has been found that in the purification of crude peroxidase solutions by adsorbents and further in the isolation of the enzyme with tannin, the loss in color is greater than the loss in enzyme. (Tannin precipitation serves to remove iron-containing, colored impurities.) On the other hand, purer peroxidase loses more in enzymic action than in color upon further purification. (Here the color of the inactive, decomposition products serves to check the retrogression of color.)

Iron Content.—Further new analytical discoveries concern the problem of the iron content of the peroxidase. In the researches of Gola [1] as well as those of Willstätter and Stoll,[2] the assumption was advanced and experimentally supported that the iron, which accompanies the peroxidase in all purification processes, is an integral constituent of the enzyme molecule. Analysis of the purer peroxidase preparates, however, has not confirmed the relation which appeared to exist between the iron content and the enzymic action. It is true indeed that iron compounds are obstinately associated with the enzyme, and accumulate at times in the course of the purification more plentifully than the peroxidase. But in the adsorption on kaolin from dilute solution and particularly in the precipitation of the peroxidase with tannin, a means has been found for deciding the question of the iron content. Elimination of the iron-containing foreign substance by tannin has been carried so far that a preparate concentrated 5-fold exhibited even a 7-fold less iron content, namely, 0.06 per cent. Hence the iron is without significance for the constitution and color of the peroxidase.

Peroxidasic Action of Oxyhaemoglobin.—The problem, much discussed in the literature, whether the blood-coloring matter, oxyhaemoglobin, possesses peroxidasic action, has been settled in the affirmative by Willstätter and Pollinger.[3] This assumption, which apparently was first suggested in the researches of Wolff and de Stoecklin,[4] has been extended in a quantitative direction

[1] R. Acc. Linc. (5) **24** I, 1239; II, 289 (1915); **28**, II 393 (1919).

[2] A. **416**, 21 and particularly p. 60 (1917–18).

[3] H. **130**, 281 (1923). See also the subsequent communication of R. Kuhn and L. Brann (B. **59**, 2370 [1926]) in which varying degrees of peroxidase activity are reported in oxyhaemoglobin and its different degradation products. Under some conditions a complete absence of catalase activity was obtained.

[4] C. r. **151**, 483 (1910); Ann. Inst. Pasteur **25**, 313 (1911).

by Willstätter and Stoll by the discovery that the oxyhaemoglobin differs from plant peroxidase in the order of magnitude of its peroxidasic action, that is, its action is relatively feeble. Quantitative study of the peroxidasic action of the blood coloring matter showed that the action observed in the crude dyestuff preparates remained up through the purest crystallizations. This was true even after complete separation of the catalase. (The methods for determination of plant peroxidases were used in these studies.) The catalytic function of the oxyhaemoglobin, whose action, contrary to that of the plant peroxidase, increases pronouncedly with rising hydrogen peroxide concentration, is found to be of varying magnitude in different kinds of animals.

While the activity of the coloring matter from the blood of different animals appears to be dependent in all cases upon the concentration of the hydrogen peroxide—whose distribution between the solution and the particles of coloring matter follows therefore in the same manner—the quantitative capacity of the separate haemoglobins is different and the activation of the hydrogen peroxide follows in unequal measure.

These gradations are based on the peroxidasic action of the iron-containing, dyestuff groups, that in different kinds of animals are united with different globin complexes; they accordingly afford a simple example for the influencing of the specifically active group of an enzyme in relation to its affinity by its association with the colloidal bearer. It is evident from this model that the specific reaction capacity of an enzymic substance may be influenced not only by the nature of the effective grouping, but also by differences in the constitution of the colloidal bearer. Such a model is based on the hypothesis developed by Willstätter, Graser and Kuhn,[1] that an enzyme consists of a colloidal carrier-complex and a purely chemically active group.

[1] H. **123**, 1 (1922).

VII. OXYDASES

Our knowledge as to the essential nature of biological oxidation processes is still very inexact, although a great number of theoretical and experimental studies have been directed toward the elaboration of this group of phenomena. None of the views expressed regarding the mechanism of oxydasic actions appears to be sufficiently well established to explain all the phenomena observed. It may suffice for the limits of this book to summarize the present state of this question and to state the three most important modes of explanation, the oxidation theories of Bach and of Warburg and the dehydrogenation theory of Wieland.

Mechanism of Biological Oxidations According to Bach.— The views developed by Bach [1] for the explanation of biological oxidations are based on the conceptions advanced by Engler [2] of the nature of catalytic oxidations in general. The conception is that the catalytic oxygen transfer takes place in the presence of an easily oxidizable substance A, the "auto-oxidizer," and of a more difficultly oxidizable substance B, the "acceptor." The auto-oxidizer enters into reaction with molecular oxygen with formation of a peroxide $A\begin{matrix}O\\|\\O\end{matrix}$, which contains the oxygen in active form. Upon retrogression of the peroxide to A, the oxidation from 2 B to 2 B O is more easily effected. In this sense, according to Bach, the oxidation effects in living organisms are to be ascribed to the cooperative action of two enzyme groups, the "oxygenases" and the "peroxidases." The first of these takes up oxygen in the peroxide form, thereby establishing a condition for the action of the peroxidasic components, which thereupon complete the true oxidation by transferring the peroxide oxygen to oxidizable substances, with regeneration of oxygenases. The

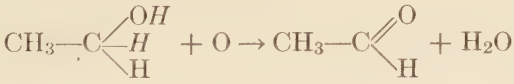
[1] A. Bach and R. Chodat, Soc. Biol. **83**, 1544 (1920); Arch. int. de Physiol. **18**, 403 (1921).

[2] See Engler and Weissberg, Kritische Studien über den Vorgang der Autoxydation. Braunschweig 1904.

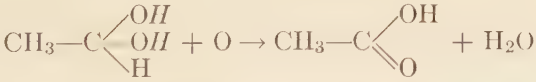
latter process takes place either directly through the splitting off of oxygen or by way of the transformation of the organic peroxide with water to hydrogen peroxide.

The frequently observed reduction effects occurring simultaneously with biological oxidations are attributed to the combined action of oxygenase and of a "perhydridase."[1] The latter is supposed to activate the hydrogen in the water present, while the oxygen produced at the same time is taken up by the oxygenatic components. These theories appear appropriate as an explanation of the wide distribution and the special significance of the peroxidasic enzymes, but they are subject to the objection that they do not provide a simple and easily visualized picture of the cooperative processes of oxidation and reduction that are of common occurrence and have been attributed with probable truth to the action of one and the same enzyme.

Hydrogen Activation According to Wieland.—With a more strictly uniform viewpoint, Wieland[2] has undertaken an explanation of biological oxidations and reductions. The nature of the oxidation processes is based by him not on a participation of activated oxygen but upon an activation of hydrogen in the substrates of the oxidation. Thus the oxidation of alcohol to aldehyde, which takes place in the cell, is ascribed to the extraction of two atoms of hydrogen from the molecule of the alcohol:



Or, according to this viewpoint, another process taking place in the cell, the formation of acids from aldehydes, proceeds by dehydrogenation by way of the aldehyde hydrate:



This concept of the activation of hydrogen has received a notable experimental support through the finding that it is possible, even in biological oxidations, to replace the oxygen by other hydrogen acceptors. The oxygen takes part in the dehydrogenation reaction only in the role of a hydrogen acceptor and can be replaced by

[1] Compare A. Bach, Bio. Z. **31**, 443 (1911).

[2] B. **55**, 3639 (1922); Ergebn. d. Physiol. **20**, 477 (1922).

other hydrogen acceptors, for example, by easily reducible substances such as quinone or methylene blue. By such a process alcohol is fermented to acetic acid with acetic bacteria and also grape sugar is burned to carbon dioxide in the exclusion of oxygen, namely, in the presence of the hydrogen acceptors introduced. And in the example of acetic acid fermentation, the quantity of acid formed was found to be equivalent to the quantity of reduction product formed from the hydrogen acceptor, quinone or methylene blue. Similar correlations between the processes of dehydrogenation and of hydrogenation have been quantitatively confirmed in the action of a typical "oxydo-reductase," Schardinger's enzyme of milk. This is capable of catalyzing the reduction of aldehydes in the presence of methylene blue, or also the disproportioning of the aldehyde to alcohol and acid, the Cannizzaro reaction.

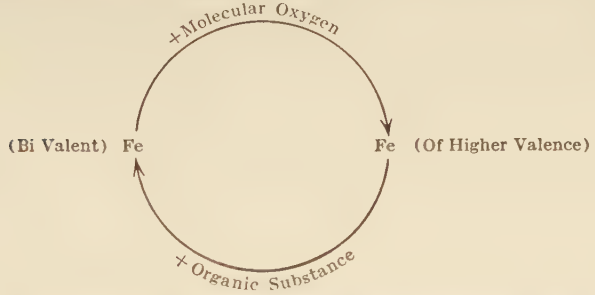
The process of oxygen hydrogenation in the respiration of the cell proceeds, according to Wieland, in the following manner: the hydrogen activated by the action of oxydase is removed from the substrate of the oxidation with correlative hydrogenation of molecular oxygen to hydrogen peroxide. The decomposition of the hydrogen peroxide may now be accomplished by the catalase with simultaneous liberation of new oxygen. In other cases the hydrogen peroxide formed serves as hydrogen acceptor for the action of peroxidasic enzymes—attributed likewise to a hydrogen activation in the substrates of the oxidation, e.g., in the phenols.

The assumptions developed by Wieland, whose experimental principles are primarily related to the oxydative degradation of sugar, gain heightened interest from the observations of Thunberg [1] regarding the mechanism of the biological oxidation of fatty acids. The experimentally accomplished dehydrogenation of succinic acid to fumaric acid by muscular tissue with methylene blue as hydrogen acceptor appears to indicate that the degradation of saturated fatty acids in the organism also follows the principle of a dehydrogenation. The first stage is the formation of unsaturated acids, from which by addition of water and further dehydrogenation, the hydrolytic splitting off of acetic acid may be induced through the intermediate stage of β-keto carbonic acid. The conception of cell respiration as a dehydrogenation

[1] Skand. Arch. f. Physiol. **25**, 37 (1911); Compare F. Batelli and L. Stern, Bio. Z. **30**, (1910).

process is further supported by the recent finding of Lipschitz,[1] that the gaseous respiration element, oxygen, in the life process of the lower organic cells, can be replaced by another sort of hydrogen acceptor, foreign to the cell, namely, m-dinitrobenzol. The m-dinitrobenzol is hydrogenated thereby to m-nitro-phenyl hydroxylamine and, like methylene blue, is capable of sustaining the respiration of surviving muscular tissue, e.g., with succinic acid.

The Iron Cycle According to Warburg.—A view contrary to that of Wieland has been advanced by Warburg [2] and supported with experimental evidence.[3] In this conception the action of activated oxygen in biological oxidations does not take place through a peroxide form but is to be considered in the sense of a surface activated by definite heavy metals, particulrarly iron. It is maintained that in respiring cells there is a cycle of the form



In this cycle molecular oxygen reacts with bivalent iron, whereby iron in a higher state of oxidation is formed. The oxidized iron reacts with the organic substance and is again reduced to bivalent iron. A study of the activity of iron in this respect and a determination of the usual iron content of cells shows that the quantity present is more than sufficient to explain the oxygen consumption incident to respiration. The assumption of such a process is based on the model experiment of the oxidation, e.g., of amino acids, by active, iron-containing carbon, whose activity as well as the process of cellular respiration suffers a specific inhibition by hydrocyanic acid. Wieland, on the other hand, ascribes the action of hydrocyanic acid on biological oxidation processes as an adverse influence on the catalase components of the oxidation system.[4]

[1] Pflüg. Arch. **196**, 463 (1922).

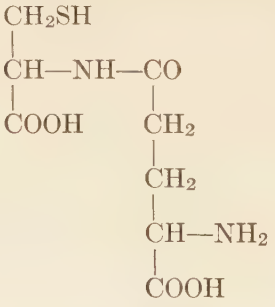
[2] Science, **61**, 575 (1925); B. **58**, 1001 (1925).

[3] B. **58**, 1001 (1925).

[4] Regarding the possibility of a combination of the theories of Wieland

Considering the still existing uncertainty concerning the mechanism of biological oxidations and concerning the nature and number of the enzymes taking part, it is obvious that our knowledge regarding the actions of the oxidation enzymes of various types of cells would still remain inadequate. Thus the earlier classification of the oxidases according to the observed actions have become wholly unreliable; such classifications involve, for example, the so-called "aerooxydases" and the hydroxydases, to which latter may be counted those enzymes having a reducing and disproportioning action. The terminology and differentiation of oxydases on the basis of their occasionally observed actions should either be established or corrected upon rational principles with the isolation of the enzymes concerned and the description of their characteristic actions. Thereupon we may succeed also in forming a decision as to the natural composition of the enzyme system taking part in biological oxidations and as to the special tasks allotted to the separate components, whose very existence, in many cases, appears to be imperfectly established.

Action of Glutathione.—Attention might be directed at this point to the noteworthy investigations of Hopkins,[1] indicating that glutathione is capable of exerting a dehydrogenating or a hydrogenating function according to the acidity of the solution. This material, glutathione, was isolated from various types of cell materials, such as yeast, muscle substance and liver and was demonstrated to be a dipeptide of glutamic acid and cystein.



and Warburg see C. Oppenheimer, Die Fermente und ihre Wirkungen, 5. Aufl., Leipzig, 1925–26, p. 1291.

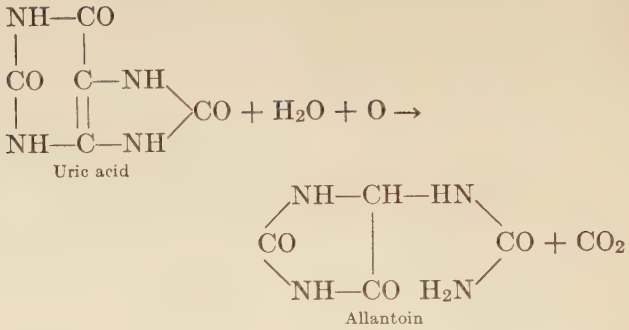
[1] Biochem. J. **15**, 286 (1921); F. G. Hopkins and M. Dixon, Jl.. Biol. Chem. **54**, 527 (1922); M. Dixon and H. E. Tunnicliffe, Proc. Roy. Soc. B. **94**, 266 (1923).

Its action as an oxydo-reductase is based on its oscillation between the cystein form with the grouping R_1-R-SH (R_1 = Glutamic acid residue, R = Cystein), which acts as a hydrogen donator and between the cystein form, the disulfide form with the grouping R_1-R-S-S-R-R_1, which acts as a hydrogen acceptor. The peptide, therefore, is capable of performing catalytic hydrogenations in the presence of other hydrogen acceptors. Its significance for the facilitation of oxidation reactions in the cells, however, is still somewhat obscure.

Purine Oxydases.—In only a few distinct cases in the literature are there special and well-established data as to the properties and action of enzymes of the oxydase group. In these cases, special oxydases or at any rate enzymes of more strongly pronounced specificity appear to be present. This holds, for example, for the so-called "purinoxydases," whose action is characterized by the oxidation to uric acid of the oxypurines formed upon the desamidation of the two amino purines, guanine and adenine. They are found predominantly in the liver and in the muscle substance of animal organisms. On the basis of the observations at hand, however, it is not yet possible to make a decision as to the specific allocation of these enzymes to the individual substrates of the purine group nor to their positive differentiation from other oxydases.

In the same manner also, the more thorough kinetic researches of Burian [1] on the oxidation of xanthine to uric acid by liver extracts, in which a further transformation of the uric acid was also observed, permit no decision as to the specific action of the oxydases investigated. It appears to be still uncertain whether the destruction of the uric acid observed is to be ascribed to the action of a special enzyme. Further, it is uncertain whether the frequently differentiated "uricase action" is to be ascribed to a specific oxydase of this group as concerns its oxydative partial process. This action consists of the transformation of uric acid to allantoin, which involves a hydrolytic and an oxidative enzymic process.

[1] H. **43**, 497 (1905).



Tyrosinase.—Also another special oxidase, "tyrosinase," must be mentioned here, which is widely distributed in animal and plant organisms. Its action consists in the oxidation of tyrosine, p-oxyphenyl-amino propionic acid, or of the derivatives of this amino acid to a red and further to a black pigment, the so-called melanine.

The formation of melanine by the tyrosinase of the potato, according to the researches of Haehn,[1] requires the presence of definite activators. This has been confirmed in a more recent work by Raper and Wormall,[2] who analyze the reaction into 3 stages: the enzymic formation of a red oxidation product, the spontaneous transformation of this product into a colorless substance, and finally the enzymically accelerated oxidation of the latter substance to melanine. The first partial process is measured by the disappearance of the tyrosin and its velocity is found to be greatest in alkaline reaction; the reaction is of a mono-molecular type, giving a true logarithmic curve when values of the concentration of tyrosine are plotted against the time, and also giving values for the velocity coefficient that are approximately constant throughout the reaction. The constitution of the intermediary and final products involved have been further elucidated by Raper and co-workers.[3]

[1] B. **52**, 2029 (1919); Bio. Z. **105**, 169 (1920).

[2] Biochem. J. **17**, 454 (1923).

[3] H. S. Raper and A. Wormall, Biochem. J. **19**, 84 (1925); F. C. Happold and H. S. Raper, Biochem. J. **19**, 92 (1925); H. S. Raper and H. B. Speakman, Biochem. J. **20**, 69 (1926); H. S. Raper, Biochem. J. **20**, 735 (1926); **21**, 89 (1927); C. E. M. Pugh and H. S. Raper, Biochem. J. **21**, 1370 (1927).

VIII. FERMENTATION ENZYMES

The Enzyme Complex "Zymase."—The process of alcoholic fermentation, the splitting of sugar into alcohol and carbon dioxide by the enzyme complex "zymase," may well be regarded as the first enzymic reaction to be investigated and practically employed. It plays a highly important role in the metabolism of a number of aerobic fungi, particularly of the species *saccharomyces*, the yeasts. The earlier investigators considered that this reaction was connected with the vital activity of microorganisms and our fundamental knowledge of modern enzyme chemistry was obtained by the description of this reaction, given in the classic investigations of Buchner [1] with respect to the separation of zymase fermentation from the life process of the yeast cell. In the preparative isolation of the zymase, however, no essential progress has been made beyond the well-known Buchner process, the production of a cellular press-juice following a mechanical destruction of the cell structure. Newer processes for the purification of enzymic substances, such as adsorption methods, have not yet been systematically applied to the fermentation enzymes. On the other hand, signally important information has been gained with respect to the reaction mechanism of alcoholic fermentation and the conditions under which it proceeds.

The Heat Resistant "Co-Zymase."—One of the principal observations, of general significance in the investigation of the composition of enzymic reaction systems, was the discovery by Harden and Young [2] of a specific heat-resistant activator of alcoholic fermentation in the yeast press-juices, "co-zymase"; in the same respect are to be mentioned the special activations of fermentation velocity by the addition of phosphoric acid salts, described by the same investigators as well as previously by

[1] E. Buchner, H. Buchner and M. Hahn, Die Zymasegärung. München, 1903.

[2] Proc. Roy. Soc. B. **77**, 405; **78**, 368 (1906).

Wróblewski [1] and by Iwanoff.[2] The special effect of phosphates, closely connected with the significance of Harden's co-enzyme and dependent on the intermediate formation of sugar-phosphoric acid ester, will be more extensively considered in the discussion of the fermentation scheme.

Our knowledge of the special properties of co-zymase has been extensively advanced by the newer investigations of Euler and Myrbäck.[3] Without the presence of this activator in washed-out dry yeast, for example, fermentation capacity in general is not observed, according to Harden and Young; further, the specific action of this activator cannot be replaced by other materials which increase the fermentation velocity of living yeast, such as the anti-neuritic vitamine. Its solutions, which are obtained by extraction of killed or dried yeast with water, have been raised to a high degree of purity by Euler and Myrbäck, namely, to a concentration of about 200-fold; their method consists in the use of various precipitation reagents as well as adsorption by alumina. It has not yet been possible, however, to make a positive chemical characterization of the activator. Raymond and Winegarden [4] have repeated the procedures developed by Euler and Myrbäck and have further tested several other methods for purification of co-zymase, but their preparates were much less active than those of Euler and Myrbäck.

Kinetics of Alcoholic Fermentation.—Measurement of the reaction course of alcoholic fermentation is commonly based on the volumetric or gravimetric determination of the carbon dioxide evolved. Thus fermentation action is expressed, according to the proposal of Willstätter and Steibelt,[5] by the "half-fermentation-time"—the number of minutes required for the liberation of 50 per cent of the theoretical carbon dioxide quantity under given normal conditions.

It is worthy of note that great differences are presented by the kinetics as well as the *p*H dependence of the fermentation with fresh and with dried yeast. Thus the phenomenon, already

[1] Zbl. f. Physiol. **13**, 284 (1899).

[2] H. **50**, 281 (1907); Zbl. f. Bakt. **24**, (1909).

[3] H. **131**, 179 (1923); **133**, 260 (1923–24); **136**, 107; **138**, 1; **139**, 15, 281 (1924).

[4] J. Biol. Chem. **74**, 175 (1927).

[5] H. **115**, 211 (1921).

observed by Harden and Young [1] of "Induction"—the spontaneous initiation of the fermentative action after the lapse of a definite induction period, which is not perceived in fresh yeast—is to be ascribed according to Meyerhof [2] and according to Euler and Myrbäck, to an insufficient formation of sugar-phosphoric acid ester in the first stages, since in the presence of this substance the fermentation begins at once. This is to be taken into consideration in the quantitatve determination of the fermentative capacity of dry yeast. In other investigations this phenomenon is regarded as a regeneration of the yeast cells.[3]

The differences observed in the kinetics of the two kinds of enzyme materials may be attributed in part to a varying dependence upon the hydrogen number, its optimal region, according to Euler and Heintze,[4] extending for fresh yeast from pH = 3 to 7.5 but for dry yeast only from pH = 6.2 to 6.8. For the fermentative action of fresh yeast, according to Slator,[5] a proportionality exists between the yeast quantity and the initial velocity over a wide range; and, according to the investigations of Tammann,[6] equal quantities of carbon dioxide are evolved in equal times with this material. With dry yeast, according to Herzog,[7] Gromow and Grigoriew,[8] as well as Euler,[9] neither the relations between the yeast quantity and the reaction-velocity nor the reaction process can be positively characterized. For the quantitative determination of zymase and its co-enzyme, according to Euler and Myrbäck, we must further take into consideration that the combination of these two substances appears to be dissociated in considerable measure and that most yeasts contain less activator than would correspond with their enzyme content. The true measure of the zymase quantities must be referred, accordingly, to the maximal fermentation velocity exhibited after the addition

[1] Compare A. Harden, Alcoholic Fermentation. 3d Edition 1923, p. 39.

[2] H. **101**, 165; **102**, 1 and particularly p. 3 (1918).

[3] See E. Abderhalden, Fermentf. **5**, 89, 110, 255, 273 (1922); E. Abderhalden and S. Glaubach, Fermentf. **6**, 143, 149, 162 (1922); H. Sobotka, H. **134**, 1 and particularly p. 9. (1923–24).

[4] H. **108**, 165 (1919).

[5] Soc. **89**, 128 (1906); **93**, 217 (1908).

[6] Ph. Ch. **3**, 25 and particularly p. 36 (1889).

[7] H. **37**, 149 (1903).

[8] H. **42**, 299 (1904).

[9] H. **45**, 420, and particularly p. 445 (1905).

of excess activator, namely, of boiled yeast juice. Hence it appears more appropriate not to base the determination of the zymase quantities and fermentative capacity upon the "half-fermentation-time," but upon the initial velocities of the fermentation; the results are not affected by the disturbances which occur in the later stages of the process of fermentation or as a result of yeast propagation.

Adaptation of Zymase to Various Substrates.—Many observations concerning the selective action of zymase have been assembled. The researches of Herzog and Saladin[1] have shown that the yeasts, which ferment the monosaccharides, glucose, fructose and mannose with nearly equal velocity, undergo essential displacements in the relations of individual fermentation velocities when they are dried with acetone. The phenomenon first described by Armstrong,[2] that most yeasts, which are incapable of fermenting galactose, acquire the power of galactose fermentation by culturing in solutions of this substrate, is to be ascribed, according to Willstätter and Sobotka,[3] to the adaptation of the zymase complex to the new fermentation substrate. This is not in the sense of the assumption by Euler,[4] which postulates a special "galactase" which converts galactose to an active form. According to Euler and Nilsson,[5] the phenomenon of galactose habituation is observed even in sterile dry yeasts,[6] and the acquired capacity for galactose fermentation suffers no diminution even upon a change in the fermentation substrate, for example, upon returning to glucose as a substrate.[7] Meanwhile, the investigation of Willstätter and Sobotka has led to the remarkable discovery that in the mixture of two fermentation substrates the relation of the fermentation velocities differs essentially from the velocity relation of the separate fermentations. The phenomenon that an yeast, well-habituated to galactose, ferments this sugar alone more rapidly than glucose, but that in a mixture of the two sugars, on the con-

[1] H. **73**, 263 (1911).

[2] Proc. Roy. Soc. B. **76**, 600 (1905).

[3] H. **123**, 176 (1922).

[4] Chemie der Enzyme, 2. Aufl., 1 Teil, München und Wiesbaden 1920, p. 293.

[5] H. **143**, 89 (1925).

[6] Compare the contradictory observations of N. L. Söhngen and C. Coolhaas, Zbl. f. Bakt. **66**, II 5 (1925).

[7] H. v. Euler and Th. Lövgren, H, **146**, 44 (1925).

trary, the glucose is preferred, indicates that the fermentations of glucose and galactose are parallel processes that coincide in a later stage of the fermentation. In these processes, the first mutual intermediate product, whose decomposition conditions the reaction, is formed from a decomposition product of glucose with greater velocity. Similar relations determine the selective fermentation in mixtures of glucose and fructose or of α- and β-glucose.[1]

Direct Fermentation of Disaccharides.—The theoretically important question whether the fermentation of disaccharides must be preceded by their enzymic cleavage into monoses has been decided by the researches of Willstätter.[2] Contrary to the older theories of Fischer and Lindner,[3] direct fermentability must be assumed for the disaccharides examined, maltose, cane sugar and lactose. This conclusion is derived from determinations of the fermentative capacity for malt sugar as exhibited by maltase poor yeasts, or, in other examples, from the undiminished fermentative power against cane sugar as exhibited by fungi, which have been made poor in saccharase by artificial methods. Further proof of the direct fermentation of cane sugar by yeast has been obtained by measuring the fermentation under strongly acid conditions, in which the fermentation exceeds the hydrolytic cleavage of the disaccharide. In the case of maltose, most favorable fermentation conditions occurred at an acidity (pH 4.5) that practically excludes the action of maltase.

Mechanism of Alcoholic Fermentation and Significance of Phosphoric Acid.—Regarding the mechanism of alcoholic fermentation, the extensive researches of Neuberg[4] above all have yielded valuable conclusions. According to Neuberg's theory, a substance with three carbon atoms, methyl glyoxal CH_3-CO-CHO, must be regarded as a primary cleavage product of the hexoses; this appears to constitute an essential intermediate stage of sugar degradation. It must be assumed therein that

[1] Compare R. Willstätter and H. Sobotka, H. **123**, 164 (1922).

[2] R. Willstätter and W. Steibelt, H. **115**, 211 (1921); R. Willstätter and E. Bamann, H. **152**, 202 (1926) (Maltose); R. Willstätter and G. Oppenheimer, H. **118**, 168 (1921–22) (Lactose); R. Willstätter and Ch. D. Lowry jr., H. **150**, 287 (1925) (Cane-sugar).

[3] B. **28**, 984, 3034 (1895); E. Fischer, H. **26**, 60 and particularly p. 72 (1898).

[4] B. **55**, 3624 (1922).

the decomposition of the hexose molecule proceeds by way of its esterification with phosphoric acid, especially since the researches of Harden and Young,[1] as well as those of Euler and Johansson [2] have shown the equivalence between the quantity of esterified phosphate and the liberated carbon dioxide. Also, according to the observations of Euler and Myrbäck,[3] the conjunctive action of the co-zymase is to be referred to this particular reaction stage of fermentation, the esterification of hexose with phosphoric acid. Concerning the further course of the degradation of the hexose molecule into substances of the three carbon series, however, there are no certain bases of information.

Formation of Lactic Acid.—Neuberg states that the transformation of methyl glyoxal can take place, under certain conditions, by the attachment of water and internal disproportioning to lactic acid, according to the reaction

$$CH_3—CO—CHO + H_2O \rightarrow CH_3—CO—CH(OH)_2$$
$$\rightarrow CH_3—CHOH—COOH$$

This process takes place above all in the carbohydrate degradation in animal muscle, which bears a relation to fermentation, and is also evidenced in the action of the lactic-acid-forming bacteria. With respect to the latter, the researches of Virtanen [4] have proved that here likewise the conjunctive action of the co-zymase is indispensable; the degradation proceeds in this case also by way of the hexose-phosphoric acid ester stage. The close relationship of carbohydrate degradation in the plant and animal organisms, at least for the first stages, is demonstrated by the important researches of Meyerhof,[5] reporting the presence of co-zymase in the boiled juice of the muscular system. This has been confirmed in the quantitative comparisons made by Euler and Myrbäck [6] of the action of the activators from muscle and from yeast. The analogy of the reaction courses in the two cases is exhibited in the coincidence of the primary reaction product, hexose-phosphoric

[1] Proc. Roy. Soc. B. **80**, 299 (1908); **81**, 336 (1909).
[2] H. **85**, 192 (1913).
[3] H. **139**, 15 (1924).
[4] H. **138**, 136 (1924); **143**, 71 (1924–25).
[5] H. **101**, 165; **102**, 1 (1918); Pflüg. Arch. **170**, 867 (1918).
[6] H. **133**, 260 and particularly p. 274 (1923–24).

acid ester. This product was isolated by Embden [1] from muscle press-juice, and by Harden and Young [2] as well as by Neuberg [3] from yeast, and has been characterized as fructose-diphosphoric acid (lactacidogen).

Formation of Pyro-racemic Acid and Acetaldehyde.—The normal transformation of methyl glyoxal taking place in alcoholic fermentation, however, according to Neuberg, proceeds through the dehydrogenation of its hydrate to pyro-racemic acid, by the equation

$$CH_3—CO—CH(OH)_2 \rightarrow CH_3—CO—COOH + H_2$$

The hydrogen formed is taken up by a substance produced in the further course of the reaction, acetaldehyde. Pyro-racemic acid, as Neuberg has shown, is smoothly and easily fermented, carbon dioxide being split off by the action of a carboxylase. The latter enzyme always accompanies zymase and possesses in general this capacity for splitting off carbon dioxide. The transformation of pyro-racemic acid into acetaldehyde according to the equation

$$CH_3—CO—COOH \rightarrow CH_3—CHO + CO_2$$

furnishes at the same time the hydrogen acceptor required for the dehydrogenation of the methyl glyoxal. The dismutation of the two aldehydes, methyl glyoxal and acetaldehyde, is now accomplished with the formation of pyro-racemic acid and ethyl alcohol:

$$CH_3—CO—CH(OH)_2 + CH_3—CHO \rightarrow$$
$$CH_3—CO—COOH + CH_3—CH_2OH$$

This reaction proceeds as a result of the constant new formation of acetaldehyde from pyro-racemic acid, as a continuous process; and consequently in the total equation of alcoholic fermentation:

$$C_6H_{12}O_6 = 2CO_2 + 2CH_3—CH_2OH$$

ethyl alcohol and carbon dioxide appear as the only products (first fermentation form).

These assumptions by Neuberg regarding the mechanism of alcoholic fermentation, that the intermediate products, pyro-racemic

[1] H. **93**, 1, 54, 124 (1924) as well as later communications.
[2] Proc. Roy. Soc. B. **82**, 321 (1910).
[3] Bio. Z. **88**, 432 (1918).

acid and acetaldehyde play a predominant role, are substantially supported by certain experimental observations. For instance, the intermediately formed acetaldehyde can be intercepted by carrying out the fermentation in alkaline, sulfite containing solution, the reaction thereby being diverted to other paths. In place of the acetaldehyde removed from the reaction in the form of its bisulfite compound, another hydrogen acceptor appears, which is of the three carbon series and is presumably methyl glyoxal. Glycerin, as its hydrogenation product, appears among the end products of the reaction along with CO_2 and acetaldehyde:

$$C_6H_{12}O_6 = CO_2 + CH_3—CHO + C_3H_8O_3$$

(Second fermentation form)

Related to the second form of fermentation described there is a third form, which Neuberg has observed in the presence of alkaline salts, but without sulfite, the specific agent for diverting acetaldehyde. In this case the aldehyde undergoes a reproportioning to acetic acid and ethyl alcohol, so that the course of the reaction in which 2 mols of glycerine and 1 mol each of alcohol and acetic acid are formed, now corresponds to the equation:

$$2C_6H_{12}O_6 + H_2O = CH_3—COOH + CH_3—CH_2OH + 2CO_2 + 2C_3H_8O_3$$

(Third fermentation form)

Likewise in a fourth form of fermentation, the so-called butyric acid fermentation, which is effected by certain microorganisms, acetaldehyde and pyro-racemic acid, represent intermediate stages, according to Neuberg. The fixation of intermediately formed aldehydes has also been carried out in this case.

Acetic Acid Fermentation.—It may be mentioned here that Neuberg and Windisch [1] have also demonstrated a similar disproportioning process for the mechanism of another fermentation phenomenon, the so-called acetic acid fermentation or the biological oxidation of ethyl alcohol to acetic acid by means of acetic acid bacteria, characteristic of the above-described fermentation forms. The bacteria of the acetic acid fermentation, which are not capable of fermenting sugar, perform the oxidation of alcohol to acid in two different stages: through the dehydrogenation to acetaldehyde and through the subsequent dismutation of the

[1] Naturw. **13**, 993 (1925).

aldehyde into acetic acid and alcohol. Neuberg and Windisch have derived this conclusion from the observation that acetaldehyde, and also pyro-racemic acid, after decarboxylation to aldehyde, do not undergo a direct oxidation but a disproportioning to acid and alcohol. This fact can be demonstrated in the exclusion of oxygen.

Synthetic Fermentations.—The significant considerations of Neuberg regarding the mechanism of alcoholic fermentation have been further expanded by the description of a new type of enzymic action among the fermentation agents, the participation of which in the fermentation process indicates the possibility of a fifth form of fermentation. This type of action is characterized by the synthesis of carbon-carbon linkages, resulting in the uniting of two aldehyde molecules to form a ketone alcohol upon addition of aldehydes to a fermenting sugar solution. For example, when benzaldehyde is added, phenyl acetyl carbinol is formed by condensation with the acetaldehyde produced in the fermentation, the reaction being:

$$C_6H_5—CHO + CH_3—CHO \rightarrow C_6H_5—CHOH—CO—CH_3$$

The synthesis of such compounds also takes place in cell-free fermentations and is therefore a purely enzymic process; according to Neuberg, the process depends on the action of a new sort of carbon to carbon-linking enzyme, the carboligase. This observation indicates that constructive processes also appear to be closely associated with the degradation reactions that take place in alcoholic fermentation. Their physiological significance is probably to be found in the synthesis of new structural materials for the cells of microorganisms.

AUTHOR INDEX

A

Abderhalden, E., 44, 48, 59, 70, 72, 115, 127, 131, 132, 156, 157, 161, 162, 166, 235
Abel, E., 216
Acklin, O., 5
Adams, M., 207
Adler, L., 153, 200, 218
Ambros, O., 151–153
Anderson, R. S., 71, 181
Armstrong, E. F., 193, 197, 236
Armstrong, H. E., 56, 117, 175, 176, 193, 195, 197
Arnheim, F., 157
Arrhenius, Sv., 42, 44, 131
Arthus, M., 148
Asada, K., 126
Auld, S. J. M., 195

B

Bach, A., 211, 217, 226, 227
Baker, J. C., 83, 202, 207
Baldwin, M. W., 13, 200, 201
Bamann, E., 71, 96, 116, 182, 183, 186, 189–193, 237
Bancels, L., 126
Barendrecht, H. P., 123, 175
Barthel, Chr., 5
Batelli, F., 214, 228
Baur, E., 4, 5
Bayliss, W. M., 44, 47, 48, 59, 126, 141
Bechold, H., 18
Benjamin, H. S., 175
Bernard, Cl., 180
Berthelot, M., 2
Bertrand, L. G., 199
Berzelius, J. J., 3
Biedermann, W., 62, 200
Bien, Z., 106
Bierry, H., 17, 61, 180, 199
Blood, A. F., 149, 152
Bloomfield, G., 44
Bodenstein, M., 54, 55
Bonem, P., 170–172
Borsook, H., 136
Bouchout, E., 149
Bourquelot, E., 56–59, 189
Boynton, L. C., 209
Bradley, H. C., 16
Brahm, C., 70
Brann, L., 224
Bredig, G., 73
Brockmann, H., 162
Brown, H. T., 202
Brücke, E., 19, 94
Brunius, E., 177
Buchner, E., 3, 80, 89, 233
Buchner, H., 3, 80, 89, 233
Burian, R., 231

C

Caemmerer, G., 70
Caldwell, M. L., 206
Chittenden, R. H., 201
Chodat, R., 211, 217, 226
Chrometzka, F., 136
Chrzaszcz, T., 204
Clark, E. D., 199
Clark, M. E., 180
Clementi, A., 170
Cohen, J. B., 116
Cohnheim, J., 19, 24, 205
Cohnheim, O., 155, 164
Connstein, W., 101, 120
Coolhaas, C., 236
Corvisart, 2
Cremer, M., 59
Croft Hill, A., 53, 56, 191

Csanyi, W., 82, 195, 196, 197
Cullen, G. E., 174, 175

D

Dakin, H. D., 115, 164, 169
Damboviceanu, A., 211, 212
Danilewsky, A., 19, 94, 136, 205
Dauphinée, J. A., 171
Davidsohn, H., 12–14, 26, 27, 68, 103, 108, 129, 139
Dawson, E. R., 116
Dernby, K. G., 25, 138, 154, 157, 166
Deutsch, W., 138
Dietz, W., 54, 55
Dixon, M., 230
Djenab, K., 124
Duñaiturria, S., 144, 145
Dyckerhoff, H., 154, 155, 161–163

E

Ebstein, W., 60, 126
Edlbacher, S., 133, 170–172
Effront, J., 204
Ehrenberg, R., 4
Ehrenreich, M., 18, 19
Einstein, A., 8
Ellinghaus, J., 133
Elvove, E., 110
Embden, G., 123, 239
Emmerling, O., 56
Engler, C., 226
Erbacher, O., 96
Euler, H. von, 5, 7, 8, 25, 27, 34, 42, 45, 46, 50, 51, 57–59, 64, 67, 68, 71, 74, 76, 86–88, 123, 157, 177, 179–181, 185, 187, 188, 201, 203, 212, 214, 234–236, 238

F

Falk, G., 201
Faraday, M., 213
Fearon, W. R., 176
Felix, K., 132, 133, 165
Fermi, C., 126
Fernbach, A., 59, 86, 122, 198
Fischer, E., 13, 56, 72, 73, 132, 156, 181, 190, 191, 194, 197, 237
Fodor, A., 4, 48, 113, 157, 187
Fosse, R., 173
Frankel, E. M., 132, 149
Freudenberg, K., 122, 123
Freundlich, H., 22, 56
Fricke, R., 208
Fromme, A., 108
Frouin, A., 129
Fuchs, W., 201
Fuld, E., 126, 149
Funke, Y., 123

G

Gambarjan, St., 86
Geret, L., 153
Gesenius, H., 133
Giaja, J., 61, 199
Gjaldbaek, J. K., 59, 128, 132, 136
Glaubach, S., 235
Glendinning, A. T., 202
Gola, G., 224
Goto, K., 132
Gottschalk, A., 133
Graham, V. A., 177
Graser, J., 6, 17, 26, 42, 45, 158, 184, 225
Grassmann, W., 16, 23, 63, 96, 139, 197, 149, 151–156, 159, 161–164
Green, J. R., 117
Grigoriew, O., 235
Gromow, T., 235
Gross, G., 126
Gross, R. E., 171
Grosser, P., 123
Grützner, P., 60, 126
Grundherr, G. E., 71, 181
Guérin-Varry, R. T., 201
György, P., 174

H

Haag, W., 154, 159
Haber, F., 56
Haehn, H., 61, 199, 232
Hahn, A., 61, 200
Hahn, M., 3, 80, 153, 233
Haley, D. E., 13, 117
Hammarsten, O., 60, 94, 137, 138, 148
Hand, D. B., 177

Happold, F. C., 232
Harden, A., 64, 123, 233, 234, 235, 238, 239
Harpuder, K., 61, 200
Harteneck, A., 5, 13, 63, 71, 94, 95, 133, 141, 142, 144, 156–158
Haurowitz, F., 14, 108–110
Hedin, S. G., 23, 49, 138, 139
Heidenhain, R., 140, 141
Heintze, S., 235
Heiss, H., 216
Helferich, B., 196
Hennichs, S., 212, 214, 215
Henri, Victor, 17, 26, 41, 61, 126, 175
Henriques, V., 59, 128, 136
Hertzmann, A. B., 16
Herzfeld, E., 4, 5
Herzog, R. O., 8, 110, 128, 131, 166, 235, 236
Hesse, A. R. F., 61, 78, 145, 199, 200, 202, 203, 206, 207
Hizume, K., 204
Holmberg, O., 200, 204
Hopkins, F. G., 230
Hoppert, C., 169
Hoppe-Seyler, F., 2
Horton, E., 174–176, 193, 195, 197
Hoyer, E., 100, 118–120
Hudson, C. S., 26–28, 182, 184, 195
Hulton, H. F. E., 83, 207
Hunter, A., 171
Hussler, J., 123

I

Iscovesco, H., 17
Issajew, W., 25, 211
Iwanoff, L., 234

J

Jacoby, M., 172, 174
Jalander, Y. W., 54
Johansson, D., 238
Johnston, M. E., 110
Jonone, R., 173
Joos, B., 209
Josephson, K., 42, 51, 57, 59, 71, 76, 181, 187, 188, 195, 196, 199

K

Kaja, P., 208
Karczag, L., 123
Karrer, P., 209
Kastle, J. H., 44, 68, 110, 180
Katschioni-Walther, 134
Kawalki, W., 8
Kay, H., 176
Kendall, E. C., 44, 59, 199, 204
Kirchhoff, G. S. C., 1, 201
Kiesel, A., 170
Klein, W., 160
Kleinmann, H., 126
Knaffl-Lenz, E., 110, 111
Koelker, A. H., 70, 127
Kollmann, T., 164
Kossel, A., 164, 165, 169
Kraut, H., 21, 93, 96, 97, 111
Krieble, V. K., 73
Kröber, E., 44, 191
Kuhn, R., 6, 15, 17, 23, 26, 34, 35, 40, 42, 45, 46, 70, 71, 72, 75, 78, 113, 116, 158, 171, 179–181, 184, 189, 190, 195–197, 205, 209, 224, 225
Kühne, W., 139, 140, 156
Kütter, S., 131
Kullberg, S., 123
Kumagawa, H., 115
Künstner, G., 133, 134, 144, 145, 165
Kusenack, W., 209

L

Langley, J. N., 60
Langmuir, I., 19
Laurin, J., 34, 45, 46
Leibowitz, J., 200, 209
Levene, P. A., 124
Levison, L. A., 126
Liebig, J. von, 1, 2, 172
Lind, O., 113
Linderstrøm-Lang, H., 127, 134, 143, 146
Lindner, P., 237
Lintner, C. J., 44, 191, 208
Lipschitz, W., 229
Loeb, J., 10
Löhlein, W., 126
Loevenhart, A. S., 44, 68, 110, 114

Lövgren, St., 88, 173, 175, 176, 236
Loew, O., 210
Lowry, Ch. D. jun., 87, 183, 237
Lüers, H., 13, 44, 200, 201, 202, 204, 207, 208
Lundin, H., 153
Lyman, J. F., 13, 117

M

Mack, E., 176
Madinaveitia, A., 214
Madsen, Th., 44
Maercker, 203
Maquenne, L., 55
Margolis, M., 131
Matthews, A., 164
McGuigan, H., 201
McGuire, G., 201
Medigreceanu, F., 124
Meisenheimer, J., 86, 87
Memmen, Fr., 14, 64, 65, 69, 72, 75, 79, 102–105, 108, 109, 111, 113, 114, 116
Mendel, L., 149, 152
Menten, M. L., 26, 28, 30–34, 36–38, 40, 42
Mett, S., 126
Meyerhof, O., 113, 235, 238
Michaelis, L., 10–15, 17–19, 23, 26–28, 30–34, 36–40, 42, 43, 47, 61, 103, 129, 139, 184, 189, 200, 211, 212
Millar, R. C., 209
Mitscherlich, E., 3
Morawitz, P., 149
Morgulis, S., 211, 212
Morris, G. H., 190
Münch, H., 40, 71, 181
Mutch, N., 169
Myrbäck, K., 64, 67, 68, 234, 235, 238

N

Nasse, O., 199
Nelson, J. M., 28, 30, 31, 44, 71, 181
Neuberg, C., 70, 123, 124, 168, 197, 237, 239, 240, 241
Nicloux, M., 44, 118
Nilsson, R., 88, 236
Noback, C. V., 177
Nordefeldt, E., 59
Northrop, J. H., 16, 125, 130, 139, 140, 143, 152, 166

O

Oeholm, L. W., 8, 9
Ohlsson, E., 203
Ohta, K., 196
Olsson, U., 51
Onodera, N., 174
Oppenheimer, C., 56, 110, 132, 166, 197, 230
Oppenheimer, G., 90, 189, 192, 194, 195, 197, 237
O'Sullivan, C., 27, 179

P

Pagès, 148
Paine, H. J., 195
Palitzsch, S., 126, 139
Parastschuk, S. W., 94
Papadakis, Ph., 181
Pasteur, L., 2, 172
Pawlow, J. P., 62, 84, 85, 129, 137, 294
Payen, A., 1
Pechstein, H., 14, 38, 39, 40, 61, 200, 211, 212
Peirce, G., 68, 111
Pekelharing, C. A., 106, 135
Perlzweig, W. A., 11
Persiel, H., 143
Persoz, J., 1
Petrou, W., 108, 110
Pincussohn, L., 70
Platt, B. S., 116
Pollak, A., 198
Pollinger, A., 21, 82, 91, 219, 222–224
Pottevin, H., 53, 54, 106, 122, 203
Pringsheim, H., 200, 201, 203, 205 209
Pugh, C. E. M., 232

R

Racke, F., 20, 23, 80, 90, 179, 181, 182, 184
Raper, H. St., 232
Rauchalles, G., 51, 163
Raymond, A. L., 234
Reiser, A., 201
Riehmann, K., 219
Ringer, W. E., 135, 139, 200
Robertson, T. B., 136
Rogozinsky, F., 152, 164
Rona, P., 13, 18, 39, 69, 103, 106, 116, 126, 136, 157, 174, 189, 198, 211, 212
Rosenheim, O., 106
Rosenthaler, L., 59, 73
Röthler, H., 170, 171
Rothstein, M., 15
Rowland, S., 138
Rubenbauer, H., 111

S

Saladin, O., 236
Salkowski, E., 153
Sawjalow, 136
Schaeffer, G., 17
Schäffner, A., 23, 96, 132, 146, 147, 156, 157, 158, 160, 164
Scheele, K. W., 122
Schepowalnikow, N. P., 62, 140
Schlätter, G., 4, 5, 159, 160
Schlesinger, M. D., 200, 203–205, 208
Schmalz, K., 201
Schmidt, A., 148
Schmiedeberg, O., 83, 168
Schneider, K., 68, 87, 112, 182, 183, 185–188
Schrumpf, P., 135
Schubert, M., 31
Schudel, G., 199
Schütz, E., 28, 130
Schütz, J. H., 131
Schwann, Th., 2, 128
Schweigart, H., 61, 199
Seifert, K., 209
Sellner, E., 204, 208
Semper, L., 86
Senter, G., 68, 212, 214
Sherman, H. C., 13, 44, 59, 112, 199–208
Sickel, H., 115
Sigmund, W., 117
Simons, E., 133, 134, 147, 164
Sjöberg, K., 201
Sjöqvist, J., 131
Slator, A., 235
Smorodinzew, J. A., 168
Sobotka, H., 46, 72, 88, 113, 189, 190, 195, 196, 235–237
Söhngen, N. L., 236
Sörensen, S. P. L., 10, 11, 13, 127, 128, 129, 134, 211
Speakman, H. B., 232
Spiro, K., 149
Spriggs, E. J., 131
Stade, W., 108
Starling, E. H., 141
Staub, M., 209
Steche, O., 214
Steibelt, W., 90, 189, 191, 192, 195, 234, 237
Steinbeck, E., 131
Stern, L., 214, 228
Steudel, H., 133
Stoecklin, E. de, 224
Stoll, A., 121, 216, 218, 222, 224, 225
Sumner, J. B., 177
Sundberg, C., 135
Svanberg, O., 27, 50, 67, 179, 180, 185, 187, 201, 203
Syniewski, V., 207

T

Takemura, M., 169
Takeuchi, T., 173
Tammann, G., 44, 45, 112, 235
Tanberg, A. P., 208
Taylor, A. E., 54, 55, 136
Thomas, A. W., 201
Thomas, P., 13, 199–201
Thunberg, T., 228
Tompson, F. W., 27, 179
Traube, M., 2
Trigt, H. van, 200

Tsuchihashi, M., 214
Tunnicliffe, H. E., 230

U

Ueda, H., 115

V

Van Eweyk, C., 198
Van Slyke, D.D., 13, 127, 134, 137, 173–175
Van't Hoff, J. H., 53
Villars, D. S., 176
Vines, S. H., 149, 151
Virtanen, A. I., 238
Volhard, F., 108, 126
Vollbrecht, E., 122, 123
Vosburgh, W. C., 28, 30

W

Waentig, P., 214
Walbum, L. E., 44, 126, 139
Waldschmidt, W., 126
Waldschmidt-Graser, J., 83, 116, 142, 168, 169
Waldschmidt-Leitz, Ernst, 5, 7, 13, 14, 20, 21, 23, 51, 71, 75, 78, 79, 83, 90, 92, 94–96, 99, 102, 104, 105, 107, 117, 119, 120, 128, 132–134, 138, 139, 141–147, 151, 156–160, 162–165, 168, 169, 199, 200, 202, 203, 206, 207
Walton, R. P., 51, 198, 201
Warburg, O., 69, 115, 226, 229, 230
Wartenberg, H., 120
Wasteneys, H., 136
Wasmund, W., 13, 44, 200–202
Wassermann, W., 20, 21, 185
Weber, H., 133, 217, 218
Weissberg, 226
Went, F. A. F. C., 86
Wenzel, E., 21, 93, 183, 186, 188
Wester, D., 174
Wiechowski, W., 169
Wieland, H., 210, 211, 226, 227, 228, 229
Wilhelmy, W. F., 25
Willstätter, R., 5–7, 14, 16, 17, 20, 21, 23, 26, 42, 45, 49, 61, 63–67, 69, 71–80, 82, 83, 87, 88–92, 94, 96, 97, 99, 102–109, 111–117, 119–121, 128, 134, 137, 143–145, 149, 151–154, 155, 158, 168, 169, 178–207, 214, 216–219, 222–225, 234, 236, 237
Windisch, F., 240, 241
Winegarden, H. M., 234
Wittich, von, 91
Wöhler, 1
Wohlgemuth, J., 85, 198
Woker, G., 50
Wolff, J., 59, 198, 224
Wormall, A., 232
Wortmann, J., 85
Wroblewski, A., 234
Wurtz, A., 149

Y

Yalander, Y. M., 118
Young, W. Y., 64, 123, 233, 234, 235, 238, 239

Z

Zacharias, 13, 173, 174
Zinsser, A., 108

SUBJECT INDEX

A

Acetaldehyde, 239
Acetic acid fermentation, 240–241
Acetophenone, 113
Adenine, 231
Adsorbates, complex, 65
Adsorption, classification, 19
Adsorption curves, 21–22, 92–93
Adsorption value, 21
Aerooxydases, 230
Agmatine, 172
Alcohol, as stimulant of acid functions, 128, 151, 221
  oxidation to aldehyde, 227
Aldehyde, oxidation to acid, 227
Alkaloids, inactivating effect, 69
Allantoin, 169, 231–232
Almonds, emulsin, 193–196
  melibiase, 197
Alpha-amylase, 71, 205
Alumina gel, adsorptive action, 18–23, 92, 106, 144–146, 151–159, 177, 184–193, 204–208, 214–215, 221–224, 234
  types, 97, 145–146
Amino groups, liberation in proteins, 131–135
Amino nitrogen, determination, 127
Amino-acylases, 81, *168–177*
Ammonium sulfate as precipitant, 91
Amygdalin, hydrolysis, 112, *193–196*
Amyl butyrate, hydrolysis and synthesis, 55
Amylase, 1, 51, 61, 78, 82, 85, 92–96, 189, *198–208*, 214
  action on yeast carbohydrates, 182
  adsorption, 92–96, 205–207
  chemical composition, 205–207
  complement, 202, 204
Amylase, historical, 1
  inhibition, 51
  rate of secretion by pancreas, 85
  salt-activation, 61, 199–204
Amylase unit, 78, 203
Amylobiase, 203
Amylose, 202
Amylopectin, 202
Anti-enzymes, 69
Arbutin, 195
Arginase, *169–172*
Arginase unit, 171
Arginine, 165, 169–172
Aspergillus niger, inulase, 209
  tannin, 122
Aspergillus oryzae, amylase, 201, 208
  See also Taka-Diastase
  histozyme, 168
  invertase, 180
  lipase, 115
  sulfatase, 124
Auto-oxidizer, 226
Auxo-ureases, 174

B

Bacteria, enzyme formation, 85, 240
Benzoyl formic acid ester, 113
Beryllium hydroxide, adsorptive action, 207
Beta-amylase, 71, 205
Beta-glucosidase unit, 195–196
Beta-glucosidases, 193–196
Biuret reaction, 156
Blasto-lipase, 120
Blood catalase, 213–215
Blood coagulation, 148–149
Bromelin, 151–153
Buffers, 10–11
Butyric acid fermentation, 240–241
Butyrin unit, 105

C

Calcium phosphate, adsorptive action, 214
Cannizaro reaction, 228
Carboligase, 241
Carbohydrases, 81, 178–209
Carboxyl groups, determination, 127–128
  liberation in proteins, 131–137
Casein, 126, 133, 134, 144, 147, 155, 156, 164
  flocculation, 137, 148
Casein as elutrient, 146
Catalase, 13, 26, 45, *210–215*, 228
  heat inactivation, 45
  kinetics, 26, 211–213
  optimum *p*H, 13, 211
Catalase unit, 213
Cellobiase, 196
Cellobiose, 209
Cellulase, 208–209
Chimosin, 138
Chlorophyll, extraction, 82, 121–122
Chlorophyllase, 121–122
Cholesterin, adsorptive action, 99, 107
Clupein, 152–164
Coadsorbents, 23
Coelutrients, 23
Co-enzymes, 174
Colloidal carrier, 6, 225
Color, relation to enzyme activity, 223
Complex adsorbates, 65, 102
Co-zymase, 64, 233–241

D

Decomposition theory, 2
Depsides, 122–123
Dextrins, 198–208
Dialysis, 89, 91, 208, 219
Diastase, *See Amylase*
Diastatic after-effect, 201

E

Edestin, 126
Egg albumin, 133, 155
Electro-dialysis, 208
Elution, 23, 144–146
Emulsin, 1, 44, 56–59, 82, 112, *193–196*
  extraction, 82, 196
  heat inactivation, 44
  historical, 1
  hydrolytic and synthetic equilibria, 56–59
  induction period, 112
  temperature coefficient, 44
Endo-enzymes, 80
Enterokinase, 61–64, 79, 93, *140–148*
Enzymes, activation, 60–69, 149–151, 199–204
  aldehyde nature, 50–51, 163
  catalytic activity compared to hydrogen ions, 46
  chemical composition, 4, 6, 108, 112, 119, 135, 184–188, 205–208, 214–215, 222, 224
  colloidal characteristics, 7–9
  combination with substrate, 23, 29–43, 47, 51, 140, 146, 174, 179–180, 200
  compensating activation, 77, 102, 109, 117, 143
  definition, 3
  diffusion velocity, 8
  distribution and occurrence, 79–80
  electrolytic character, 10–23
  equivalent weights, 67
  inactivation by heavy metal salts, 67
  inhibition, 60–69, 144, 212
  inhibition by cleavage products, 144
  liberation, 80–84
  material nature, 3–6
  measurement, 74–79
  molecular weight, 8–9
  particle size, 7
  dependence on H-ion concentration, 11–16
  precipitation, 91
  preparative technique, 74–100
  protein nature, 108, 112, 119, 135, 187

Enzymes, protoplasmic nature, 4
rate of secretion in animal organisms, 84–85
solubility, 89, 92, 99, 169, 191, 219
specificity, 70–73
synthetic action, 52–59, 136–137, 169, 176, 241
temperature dependence, 43
theory of action mechanism, 47
Enzyme creams, 100, 118–120
Enzyme-substrate compound, dissociation constant, 33, 78
Enzyme units, *74–79*, 103–105, 118, 144, 158, 171, 179, 195–196, 203, 213
Erepsin, 13, 25, 44, 51, 95–96, 134–135, 138–139, 153, *155–167*
adsorption, 95–96
kinetics, 25, 113
optimum *p*H, 13, 51, 157
temperature coefficient, 44
Erepsin unit, 158
Ester saponification, kinetics, 29, 52–56
Esterases, 81, 101–124
specificity, 114–116
Exo-enzymes, 80
Extra-cellular enzymes, 80, 83

F

Ferment concept, 1–6
"Fermentatio," 1
Fermentation, 1, 2, 233–241
Ferric hydroxide, adsorptive action, 207
Fibrin, 146–149, 152, 155
Fibrinogen, 148–149
Formol titrations, 127, 170

G

Galactose, fermentation, 122, 236
Galactosidases, 196–198
Gastric lipase, 13, 14, 108–110
Gelatine, 133, 139, 143, 147, 152, 155
Gentiobiase, 193, 196
Germinating seeds, 153, 202, 204, 207, 209
Gliadin, 147
Globin, 225
Glucosidases, 13, 188–196
Glucosides, hydrolysis and synthesis, 56–59, 188–196
Glutamic acid, 159
Glutathione, 230
Glycerine, stabilizing action, 106, 158
Glycerine fermentation, 240–241
Glycogen, hydrolysis, 198–208
Glycyl-glycine, 50, 157, 163
Guanidine derivatives, 172
Guanine, 231

H

Hämase, 214
Helicin, 194
Hemicellulases, 209
Hexose phosphate, 113, 233–241
Hexosidases, 178–198
Hippuric acid, hydrolysis and synthesis, 168–169
Histone, 133, 134, 147, 156, 165
Histozyme, 83, 168–169
extraction, 83, 169
Honey invertase, 180
Horse-radish, peroxydase, 219
Hydrocyanic acid, inhibiting effect, 69, 229
activation of papain, 149–151
Hydrogen, enzymic activation, 227–229
Hydrogen peroxide, decomposition, 210–215
Hydrogen sulfide, activation of bromelin, 151–152
Hydroxydases, 230

I

Induction period, 112–113
Intestinal erepsin, 155–167
Intestinal invertase, 178–188
Intra-cellular enzymes, 80, 116–120
Inulase, 208–209
Invertase, 12, 13, 15, 18, 25–45, 50, 67, 68, 74, 82, 85–89, 96, 98, *178–188*
adsorption, 96, 98, 183–187
chemical composition, 74, 184–188

Invertase, dissociation residue, 15
electrical migration, 18
extraction from yeast, 82, 181–183
formation in yeast, 85–89, 180
heat inactivation, 45
inactivation by heavy metal salts, 67
inactivation by organic bases, 68
inhibition, 39, 50
kinetics, 25–43, 158
measurement, 35, 178–180
optimum *p*H, 12, 13, 173
separation from maltase, 96, 186
stability, 26, 99, 178
temperature coefficient, 44
Iron content of peroxydase, 224
Iron cycle, 229–230

J

Jack bean urease, 172

K

Kaolin, adsorptive action, 18–23, 92–96, 106, 144–146, 184–187, 214–215, 221–224
Kidney, arginase, 170–171
histozyme, 168–169
sulfatase, 124
Kinase unit, 79
Kinetics of enzyme action, *24–59*, 110, 122, 130, 140, 157, 174, 189, 195, 201–203, 211–213, 232, 234–236

L

Lab, 60, 94, *129–138*, 148. *See also Pepsin*
Lactic acid fermentation, 238
Lactacidogen, 123, 239
Lactase, 196–198
Lead phosphate precipitations, 186
Leaves, amylase, 201
Lichenase, 208–209
Lienoprotease, 138–139
Lipase, 53–55, 64–66, 75, 76, 79, 85, 92–96, 98, *101–109*, *116–118*
adsorbates, 65
activation, 64–66
Lipase, adsorption, 92–96, 98
chemical composition, 108
destruction, 103
destruction by alcohol and ether, 90, 92, 106
rate of secretion by pancreas, 85
stability in glycerine solution, 106
stereochemical specificity, 109
synthetic action, 53–55
toxic susceptibility, 116
Lipase unit, 75, 79, 103–105
Liver amylase, 198, 204
Liver arginase, 170
Liver catalase, 213
Liver esterase, 110–114
Liver lipase, 72
Liver oxidase, 231
Lysine, 133

M

Malachite green, enzymic formation, 216–214
Malt amylase, 198–208
optimum *p*H, 13, 200
temperature coefficient, 44
Malt proteases, 153
Maltase, 13, 44, 90, 96, *188–193*, 237
adsorption, 96, 192–193
liberation from yeast, 90, 190–193
optimum *p*H, 13, 189, 237
separation from invertase, 96, 192–193
temperature coefficient, 44
Maltose, synthesis, 56
Mandelic acid ester, saponification, 113
Mandelic acid nitrile, enzyme synthesis, 73
Mannose, fermentation, 236
Measurement scales, 75–79
Melanine, 232
Melibiase, 196–198
Mercury salts as precipitant, 220–221
Methyl glyoxal, 237
Methyl oleate, synthesis by lipase, 53
Methylene blue, 228
Milk coagulation, 137–138
Millon reaction, 112

Mono-molecular reactions, 24–27
Muscle, co-zymase, 238
  histozyme, 168
Muta-rotation, 205

N

Nucleic acids, 124, 188

O

Ornithine, 170
Ovaries, arginase, 170
Oxydases, 81, 226–232
Oxydo-reductase, 228
Oxygen acceptor, 226
Oxyhaemoglobin, 224–225
Oxynitrilase, 193

P

Pancreas, separation of enzymes, 93–96, 144–146
Pancreatic amylase, *198–208*
  optimum *p*H, 13, 200
  temperature coefficient, 44
Pancreatic erepsin, *156–167*
Pancreatic lipase, 13, 72, *101–108*, 144
  optimum *p*H, 13, 103
  specificity, 72
  temperature coefficient, 44
Pancreatic trypsin, *139–148*
Papain, 63–64, 137, *149–151*
  activation by HCN, 63–64
Papayotin. *See Papain*
Parachimosin, 138
Penicillium glaucum, inulase, 209
Pepsin, 2, 13, 19, 28, 44, 60, 94, *129–138*, 165
  adsorption, 19, 94, 135
  historical, 2
  kinetics, 28, 130
  optimum *p*H, 13, 129
  temperature coefficient, 44
  velocity of secretion, 129
Pepsinogen, 129
Peptidases, 125–167. *See also Erepsin*
Peptide hydrolysis, kinetics, 25, 131
Peptides, 125–167
Peptones, 141, 147, 150–154, 156
Perhydridase, 227
Peroxydase, 74, 82, 89, 91, *216–225*
  color, 74, 223
  extraction, 82, 219–221
  fixation, 89, 220
  iron content, 224
  precipitation, 91, 220–221
Peroxydase unit, 218
Phäophytin, 121
Phenol carboxylic acids, 122–123
Phosphatase, 123–124
Phosphatese, 123
Phytol, 121
Phytolipase unit, 118
Pineapple protease, 151–153
Plastein formation, 136–137
Platinum, catalytic action, 213
Polyases, 198–209
Polypeptidase, 155
Polypeptides, 125–167
Potato amylase, 199
Pro-enzymes, 60, 94
Pro-kinase, 94, 142
Proline, 165
Pro-pepsin, 129
Protamines, 131, 141, 147, 156, 164–165
Proteases, 16, 44, 59, 81, *125–167*
  classification, 16, 125, 166–167
  determination of activity, 125–128
  heat inactivation, 44
  mechanism of action, 161–167
  occurrence, 81, 125–167
  synthetic action, 59, 136–137
    See also Bromelin, Enterokinase, Erepsin, Lab, Lienoprotease, Papain, Pepsin, Thrombin, Trypsin
Protein hydrolysis, kinetics, 28, 130–131
Protein metabolism, 172
Proteins, coagulation, 132
  solubility, 126, 137
Protenoids, 132
Prunasin, 194
Pumpkin protease, 152–153
Purine oxydases, 231

Purpurogallin, enzymic formation, 215–219
Purpurogallin number, 218
Pyrogallol, 216–219
Pyro-racemic acid, 239

Q

Quantity–Time–Quotient, 179
Quinone, 228

R

Raffinose, hydrolysis, 70, 72, 181, 197
Resolution of racemic amino acids, 169
Respiration enzymes, 210–211, *226–231*
Ricinus lipase, 13, 44, 84, 99–100, *116–120*
  optimum *p*H, 13, 117
  purification, 99–100
  temperature coefficient, 44

S

Saccharase, *See Invertase*
Saccharase unit, 179
Salicin, 194
Saliva amylase, 198, 205
Saliva invertase, 180
Salmine, 164
Schardinger's enzyme, 228
Schütz rule, 28–29, 130–131, 158
Scombrine, 164
Serine, 159
Serum albumin, 155
Sexual activity and arginase concentration, 170
Ship-worm, cellulase, 209
Sodium fluoride, inhibiting effect, 68
Soya bean urease, 172
Specific activation, 62–64
Specific reactive group, 6, 50–51, 92, 99, 143, 162–163, 223, 225
Spermato-lipase, 120
Spleen proteases, 138–139
Starch, hydrolysis, 71, 198–208
Stereochemical specificity, 73, 109
Stomach lipase, 13, 14, 108–110
Sturine, 164
Substrate ionization, 16
Substrates, elutriating effect, 23, 146
Succinic acid, dehydrogenation to fumaric acid, 228
Sugar beet, invertase, 180
Sugar phosphoric acid esters, 113, 233–238
Sulfatase, 123
Sulfonic acids, 127

T

Taka–Diastase, 202, 205
Tannase, 122–123
Tannin precipitation, 186, 220–225
Tartaric acids, resolution, 116
Taurine, 127
Testicles, arginase, 170
Thrombin, 148–149
Thrombokinase, 149
Thymolphthalein, 133
Time Value, 179
Triacetin, hydrolysis and synthesis, 74
Tri-butyrin hydrolysis, 103–105, 109
Triolein, synthesis by lipase, 54
Tristearin, adsorptive action, 99, 107
Trypsin, 2, 13, 44, 62–64, 85, 90, 130, 134, *139–148*, 150–151, 156, 158, 187, 196, 214
  action on almond extracts, 196
  action on catalase, 214
  action on invertase, 187
  action on yeast proteins, 90
  activation by enterokinase, 62–64, 134–135, 140–148
  adsorption, 92–96
  historical, 2
  optimum *p*H, 13, 130
  rate of secretion by pancreas, 85
  temperature coefficient, 44
Trypsin unit, 144
Tryptophane, 185, 187–188
Turnip, peroxidase, 219, 223
Tyrosine, 159, 232
  stabilizing action, 112
Tyrosinase, 232

U

Urea, 170–177
Urease, 13, *172–177*
  optimum *p*H, 13, 173
Uric acid, 169, 231–232

V

Viscose, enzymic hydrolysis, 209
Vitalistic theory, 2
Vitamine, anti-neuritic, 234

X

Xanthine, 231
Xanthydrol, 173

Y

Yeast gentiobiase, 196
Yeast invertase, 85–89, *178–188*
Yeast lactase, 197
Yeast maltase, *188–193*
Yeast melibiase, 197
Yeast proteases, 96, *153–155*
Yeast zymase, *233–241*
Yeasts, autolysis, 86–90, 182–183
  inversion capacity, 86

Z

Zein, 147
Zymase, 3, 64, *233–241*
Zymogens, 60–62

LANE MEDICAL LIBRARY
300 PASTEUR DRIVE
PALO ALTO, CALIFORNIA 94304

Ignorance of Library's rules does not exempt violators from penalties.

MAR 2 4 1973

50M—10-63—5632

LANE MEDICAL LIBRARY
STANFORD UNIVERSITY
MEDICAL CENTER
STANFORD, CALIF. 94305

QP601
W16w
1929
Waldschmidt-Leitz, E.
Enzyme actions and properties
71149
NAME
DATE DUE
Pui-Chu Chan
Britannica
OCT 20 1965
6-17-67
MAR 18 1968
MAR 24 1973